An Essential Role of Variant Histone H3.3 for Ectomesenchyme Potential of the Cranial Neural Crest

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Abstract

The neural crest (NC) is a vertebrate-specific cell population that exhibits remarkable multipotency. Although derived from the neural plate border (NPB) ectoderm, cranial NC (CNC) cells contribute not only to the peripheral nervous system but also to the ectomesenchymal precursors of the head skeleton. To date, the developmental basis for such broad potential has remained elusive. Here, we show that the replacement histone H3.3 is essential during early CNC development for these cells to generate ectomesenchyme and head pigment precursors. In a forward genetic screen in zebrafish, we identified a dominant D123N mutation in h3f3a, one of five zebrafish variant histone H3.3 genes, that eliminates the CNC-derived head skeleton and a subset of pigment cells yet leaves other CNC derivatives and trunk NC intact. Analyses of nucleosome assembly indicate that mutant D123N H3.3 interferes with H3.3 nucleosomal incorporation by forming aberrant H3 homodimers. Consistent with CNC defects arising from insufficient H3.3 incorporation into chromatin, supplying exogenous wild-type H3.3 rescues head skeletal development in mutants. Surprisingly, embryo-wide expression of dominant mutant H3.3 had little effect on embryonic development outside CNC, indicating an unexpectedly specific sensitivity of CNC to defects in H3.3 incorporation. Whereas previous studies had implicated H3.3 in large-scale histone replacement events that generate totipotency during germ line development, our work has revealed an additional role of H3.3 in the broad potential of the ectoderm-derived CNC, including the ability to make the mesoderm-like ectomesenchymal precursors of the head skeleton.

Introduction

The development of multipotent, migratory NC cells was a key step in the evolution of many of the vertebrate-specific features of the head [1]. CNC cells generate the skeleton of the face and anterior skull, as well as supporting development of the brain and the head [1]. CNC cells generate the skeleton of the face and anterior skull, as well as supporting development of the brain and the head [1].

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of those seen in (Figure 1c, 1d). These skeletal phenotypes were very reminiscent lack CNC, again confirming the CNC specificity of the head homozygous and heterozygous embryos, (Figure 1e, 1f). Due to the shared phenotypes of db1092 exhibited variable reductions of the jaw-support skeleton whereas some db1092 heterozygotes survived to adulthood, others exhibited variable reductions of the jaw-support skeleton (Figure 1e, 1f). Due to the shared phenotypes of db1092 homozygous and heterozygous embryos, “db1092 mutants” will refer to both genotypes unless otherwise stated.

We next examined whether other NC derivatives, such as pigment cells, glia, and neurons, were affected in db1092 mutants.

Each of histones H2A, H2B, H3 and H4, wrapped by two turns of double-stranded DNA. Whereas canonical H3 histones (H3.1 and H3.2) are incorporated into chromatin predominantly during replication, H3.3 is also incorporated outside of replication [16], which has implicated it in various histone replacement events including gene regulation. In mammals, H3.3 has been associated with large-scale histone replacement during the specification of primordial germ cells [17] and the inactivation of meiotic sex chromosomes [18]. However, a developmental requirement for H3.3 outside the germ line has yet to be described. Flies lacking both H3.3 genes are infertile yet largely adult viable with no specific developmental abnormalities [19,20]. Similarly, mice hypomorphic for H3.3A display growth reduction and infertility but no specific developmental defects [21]. However, the presence of multiple, identical copies of histone genes, such as H3.3, has complicated loss-of-function studies, particularly in vertebrates. Through genetic studies in zebrafish, we have identified a D123N mutant form of H3.3 that allows us to dominantly interfere with H3.3 chromatin incorporation during development. In so doing, we have found that the formation of CNC cells, and their subsequent lineage potential, are particularly sensitive to deficits in H3.3 incorporation.

Results

A dominant H3.3 mutation specifically disrupts CNC development

In an ethynitrosourea mutagenesis screen, we identified a dominant zebrafish mutant, db1092, that exhibited severe reductions in fltlα:GFP-labeled ectomesenchyme [22] at 36 hours-post-fertilization (hpf) (Figure 1a, 1b). As in other vertebrates, the facial skeleton and anterior skull of the larval zebrafish derive from CNC ectomesenchyme, with the posterior skull being of mesoderm origin [23,24]. In db1092 homozygous mutants, nearly all of the CNC-derived cartilage, bone, and teeth were lost at 5 days-post-fertilization (dpf), leaving only the mesoderm-derived skull (Figure 1c, 1d). These skeletal phenotypes were very reminiscent of those seen in foxd3; gfp2a compound mutants that completely lack CNC, again confirming the CNC specificity of the head skeletal defects in db1092 mutants [25]. db1092 homozygous larvae die by around 7 dpf, presumably due to an inability to feed. Whereas some db1092 heterozygotes survived to adulthood, others exhibited variable reductions of the jaw-support skeleton (Figure 1e, 1f). Due to the shared phenotypes of db1092 homozygous and heterozygous embryos, “db1092 mutants” will refer to both genotypes unless otherwise stated.

We next examined whether other NC derivatives, such as pigment cells, glia, and neurons, were affected in db1092 mutants. Melanophore pigment cells and their det-positive precursors were reduced in the cranial but not trunk regions of db1092 mutants, and to a lesser extent so were xanthophore pigment cells and their xdh-positive precursors (Figure 1h, 1j and Figure S1). In contrast, foxd3-positive peripheral glia (Figure 1f), neurons of the cranial ganglia (Figure S1), and the dorsal root ganglia and sympathetic neurons derived from trunk NC (data not shown) were unaffected. db1092 mutants also displayed mild heart edema, consistent with a known CNC contribution to the heart [26], but had an otherwise remarkably normal morphology at 5 dpf (Figure 1s). In summary, db1092 mutants have highly specific reductions of CNC derivatives, in particular the ectomesenchymal/skeletal components of the head.

We next used microsatellite polymorphism mapping to place db1092 within a 464 kb region on linkage group 3 which contained k3βα, one of five genes encoding identical H3.3 proteins (Figure 2). Sequencing of k3βα revealed a G to A transition in db1092 that converts aspartic acid 124 to asparagine (referred to as D123N due to cleavage of the amino-terminal methionine). Given the semi-dominant nature of db1092, we reasoned that the D123N mutation might result in a dominantly acting version of H3.3. To test this, we separately injected mRNAs encoding wild-type and D123N forms of H3.3 into one-cell-stage zebrafish embryos. Whereas wild-type H3.3 had no effect on CNC development, D123N H3.3 caused nearly identical losses of fltlα:GFP-positive ectomesenchyme (Figure 1n), CNC-derived head skeleton (Figure 1p), and cranial melanophore precursors (Figure 1r) as seen in the db1092 mutant, confirming D123N H3.3 as the causative mutation. As reported for other H3.3 genes in zebrafish [27], we found that k3βα was ubiquitously expressed starting at 4 hpf and continuing through 14.5 hpf when CNC has been specified (Figure 5). At 16.5 and 27 hpf, k3βα expression remained largely ubiquitous but was more prominent in the anterior embryo. As both the endogenous k3βα gene product, and in particular the mRNA-injected D123N H3.3, are present uniformly throughout the embryo at CNC specification stages, the remarkable specificity of the ectomesenchyme defect is not due to a preferential expression of this particular k3βα gene in CNC precursors. Instead, our data indicate that CNC and ectomesenchyme development are uniquely sensitive to altered H3.3 function.

Mutant D123N H3.3 dominantly interferes with H3.3 function through aberrant homodimer formation

We next investigated the effect of the D123N substitution on H3.3 function. When human embryonic kidney cells were transfected with FLAG-tagged wild-type or D123N H3.3, we found D123N H3.3 to be under-enriched in purified nucleosomes compared to wild-type H3.3 (Figure 4a). The D123N mutation also prevented the incorporation of H3.3 into chromatin in zebrafish embryos. Whereas mCherry-tagged forms of both wild-type and D123N H3.3 were nuclear localized during interphase, during metaphase/anaphase, when the nuclear membrane breaks down and condensed chromosomes are easily distinguished, wild-type but not D123N H3.3 co-localized with chromatin marked by a GFP-tagged H2A.F/Z histone [28]. The failure of D123N H3.3 to associate with chromatin was observed both in the eye (Figure 4b) and in the pax3:GFP-positive PNB precursors of CNC (Figure S2). Time-lapse recordings showed that mCherry-D123N-H3.3 nuclear fluorescence immediately returned upon resumption of interphase, indicating that the diffuse metaphase fluorescence of D123N H3.3 was due to a lack of chromatin incorporation and not degradation (Figure S3).
Figure 1. A dominant H3.3 mutation results in losses of CNC–derived head skeleton and pigment cells. a, b, fli1a:GFP-labeled arch ectomesenchyme (arrowheads) is greatly reduced, yet fli1a:GFP-positive endothelial cells (top) are unaffected, in both homozygous and heterozygous h3f3adb1092 mutant at 34 hpf (7/7 mutant; 0/10 wild-type). c, d, Homozygous h3f3adb1092/db1092 embryos specifically lack the CNC-derived head skeleton at 5 dpf (36/71 complete loss; 35/71 partial loss). Diagrams show the CNC-derived cartilage (blue) and bone and teeth (red), mesoderm-derived cartilage (green), pectoral fin cartilage (black), and eyes (yellow). e, f, h3f3adb1092/+ heterozygous larvae exhibit a wide range of craniofacial defects. In some cases, no defects are observed in the facial skeleton and heterozygotes are adult viable (not shown). In mild cases (e), dorsal cartilage and bone of the first and second arches are preferentially reduced, including the dorsal hyosymplectic cartilage and opercular bone of the second arch (arrow). In more severe cases (f), the cartilage and bone of the first arch and dorsal second arch are greatly reduced, with the anterior neurocranium and the posterior ceratobranchial cartilages being less affected. The frequency of skeletal phenotypes in h3f3adb1092/+
Nucleosome assembly normally proceeds through an H3–H4 heterodimer, an H3–H4/H3–H4 heterotetramer, and then the octamer. The C-terminal domain of H3 forms a 4-helix bundle with a second H3, thus promoting the association of the two H3–H4 pairs within the nucleosome. D123 lies within this domain and forms an intermolecular bond with histidine 113 (H113) of the alternate H3 [29]. In purified nuclear fractions from cultured cells, we found that wild-type H3.3 associated with both H3 and H4, reflecting the presence of heterotetramers and octamers. Remarkably, rather than blocking H3.3–H3 interactions, the D123N mutation resulted in H3.3 forming aberrant associations with H3 in the absence of H4 (Figure 4c). The dominant-negative function of D123N H3.3 would thus be explained by its ability to complex with wild-type H3.3 in the absence of H4, thus interfering with the ability of wild-type H3.3 to be incorporated into nucleosomes. Consistent with this, misexpression of D123A and H113A mutant versions of H3.3, which are predicted to fail to associate with H3, had no effect on CNC development (data not shown). Of note, we were unable to detect mislocalization of mCherry-tagged wild-type H3.3 within h3f3a/db1092 homozygotes (Figure S4), suggesting that H3.3 is not whole-scale depleted from chromatin in mutants. Thus, the dominant effects of D123N H3.3 on CNC development could be due either to a partial depletion of wild-type H3.3 from chromatin, which falls below our level of detection, or alternatively a failure to incorporate H3.3 at a particular subset of loci, such as at poised and active enhancers. Importantly though, increasing H3.3 levels by injection of wild-type H3.3 mRNA rescued the head skeletal defects of h3f3a/db1092 mutants, showing that defects are indeed due to compromised H3.3 incorporation and not neomorphic effects of mutant D123N H3.3 on unrelated pathways (Figure 5a, 5b).

Whereas misexpression of mutant D123N H3.3 results in severe CNC defects, it remains unclear whether loss of H3.3 genes can cause a similar effect. H3.3 genes are some of the most highly expressed in cells, and we were unable to generate function-blocking morpholinos that substantially reduced H3.3 levels for any of the endogenous genes (data not shown). However, injection of a morpholino that partially reduced splicing of the h3f3a gene was able to rescue h3f3a/db1092 mutants by reducing D123N H3.3 levels, though it caused no CNC defects on its own (Figure 5c–5g). Mice with hypomorphic mutations in H3.3a, one of two H3.3 genes, also do not exhibit specific CNC/craniofacial defects [21]. In one model, the dominant D123N H3.3 protein is particularly effective at depleting available H3.3 levels below the threshold

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Figure 2. Identification of the h3f3a/db1092 lesion. a, The db1092 allele was crossed to the highly polymorphic WIK strain for linkage analysis. As the db1092 mutation is semi-dominant, we enriched for putative heterozygotes by selecting for partial head skeletal loss, and events were scored as recombination only if both chromosomes displayed the wild-type WIK polymorphism. Using a set of microsatellite ‘Z’ markers spanning the zebrafish genome, we placed db1092 on linkage group 3 near Z3725 and Z20058, and subsequent linkage analysis placed it between Z63643 and Z66457. Recombinants per 1065 meioses are listed above each marker. Sequencing of 3’ UTRs identified single nucleotide polymorphisms (SNPs) that created or destroyed restriction sites between the mutant and WIK chromosomes. These SNPs (identified by their position in millions of base pairs) and Z48485 were then used to map db1092 to a 464 kb interval. b, Electrophoretograms show a G to A transition in the h3f3a gene of db1092 homozygotes. c, Schematic of the H3.3 variant histone protein encoded by h3f3a. The db1092 mutation results in a D123N substitution near the C-terminus of the core domain.
required for CNC development, with a general reduction in H3.3 nucleosome incorporation resulting in CNC defects. Alternatively, as discussed above, CNC defects may arise from defects in a particular subset of nucleosomal H3.3 incorporation. Indeed, recent studies suggest that distinct chaperone complexes load H3.3 at different types of genomic sites, and particular complexes could be differentially sensitive to dominant effects of mutant D123N H3.3 [30]. In the future, the generation of null mutants for some or all H3.3 genes should help address whether CNC development is particularly sensitive to reductions in general or specific modes of H3.3 nucleosomal incorporation, as well as revealing to what extent H3.3 is required in other non-CNC tissues.

CNC development depends on replication-independent H3.3 incorporation

Histone H3.3 can be incorporated into chromatin both during and outside of DNA replication. In contrast, canonical H3.2 differs from H3.3 at only four amino acids yet incorporates very poorly into chromatin outside of replication [16]. Hence, in order to test the role of replication-coupled loading of H3 histones in CNC development, we performed embryo-wide misexpression of a version of H3.2 carrying the same D123N mutation. As with H3.3, the D123N mutation blocked the incorporation of mCherry-tagged H3.2 into H2A.F/Z:GFP-labeled chromatin (Figure 4b). However, unlike D123N H3.3, D123N H3.2 had no effect on development of βl[a]GFP-positive ectomesenchyme or CNC-derived head skeleton (Figure 4d and data not shown). The inability of D123N-H3.2 to inhibit ectomesenchyme formation strongly suggests that CNC development relies on the unique replication-independent mode of H3.3 deposition.

H3.3 function is required cell-autonomously for CNC but not NPB gene expression

In order to understand at what level H3.3 functions in CNC development, we next examined gene expression in h3f3a\textsuperscript{d\textsubscript{1092}} embryos. NC arises at the juncture of neural and non-neural ectoderm by the combined action of multiple signals, including BMPs, FGFs, and WNTs [31], with these signals inducing a group of transcription factors with overlapping expression at the NPB. Subsequently, a subset of NPB cells upregulate an additional group of transcription factors in presumptive NC, with these cells delaminating and migrating away from the neural tube shortly thereafter. At 10 hpf, we found that the NPB expression of msxb, pax3a, zic2a and tfap2a was indistinguishable between wild-type and h3f3a\textsuperscript{d\textsubscript{1092}} embryos (Figure 6a–6d). Neural and otic placode patterning was also normal, as shown by forebrain (\textit{dlx2a}), midbrain (\textit{pax2a}), hindbrain (\textit{egr2b}), and otic (\textit{sox10}) gene expression (Figure 6m; Figure S5). In contrast, the expression of snai2, sox10, foxd3, and sox9b was lost or greatly reduced in the presumptive CNC domains of h3f3a\textsuperscript{d\textsubscript{1092}} mutants (Figure 6g–6k), as was sox10 expression in D123N-H3.3-injected embryos (Figure 6l). However, the NPB-specific expression of \textit{msxb} and \textit{pax3a} remained unaffected at these stages, indicating that the loss of CNC expression was not due to cell loss (Figure 6e, 6f). In particular, the loss of tfap2a expression in the later CNC domain (Figure 6j) but not the earlier NPB domain (Figure 6d) of h3f3a\textsuperscript{d\textsubscript{1092}} mutants highlights the selective role of H3.3 in CNC gene expression. Interestingly, h3f3a\textsuperscript{d\textsubscript{1092}} embryos had no defects in trunk NC formation as assayed by \textit{crestin} and \textit{sox9b} expression at 11.7 hpf (Figure 7), consistent with trunk NC derivatives being unaffected.
Figure 4. The dominant D123N mutation prevents chromatin incorporation and promotes the formation of aberrant H3 homodimers. 

a. Western blots show α-FLAG immunostaining of nuclear extracts or purified mononucleosome fractions from HEK cells transfected with vector alone or FLAG-tagged H3.3 (f:H3.3) vectors. Wild-type and D123N f:H3.3 proteins are expressed at equal levels in total extract, but wild-type f:H3.3 is present at much higher levels in the nucleosome fraction (consistent over three replicate experiments). α-FLAG immunoprecipitation from purified nucleosomes shows that wild-type but not D123N f:H3.3 is incorporated into nucleosomes containing H2A, H2B, H3, and H4 (consistent over three replicate experiments).

b. Confocal images from H2A.F/Z:GFP embryos expressing wild-type and D123N versions of mCherry(m)H3.3 and mCherry(m)H3.2 fusion proteins. Merged images show that whereas all H3 proteins are nuclear localized in surrounding non-mitotic cells, wild-type mH3.3 and mH3.2, but not D123N mH3.3 and mH3.2, co-localize with H2A.F/Z:GFP in the chromosomes of metaphase/anaphase cells (arrowheads) after nuclear envelope breakdown (wild-type mH3.3, 11/11 cells in 2 embryos; D123N mH3.3, 0/25 cells in 3 embryos; D123N mH3.2, 21/21 cells in 3 embryos; D123N mH3.2, 0/16 cells in 2 embryos). c. α-FLAG, α-H3 and α-H4 western blots for samples immunoprecipitated by α-FLAG from nuclear extracts of f:H3.3-transfected HEK cells. Recombinant octamer is used as a reference. Whereas both endogenous H3 and H4 co-immunoprecipitate with the wild-type f:H3.3 protein (*), H3 but not H4 co-immunoprecipitates with D123N f:H3.3 (asterisk marks the larger recombinant f:H3.3 protein). Results were consistent over three replicate experiments. d, mRNA injection of D123N mH3.3 (8/17), but not wild-type mH3.3 (8/17), wild-type mH3.2 (0/19), or D123N mH3.2 (0/18), results in loss of the CNC-derived head skeleton at 4 dpf. Scale bars: b, 10 μm; d, 250 μm.

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While it remains unclear why trunk NC is spared, the greater sensitivity of cranial versus trunk NC to defects in H3.3 function correlates with the greatly increased capacity of CNC to generate ectomesenchyme. Consistent with a direct role of H3.3 in CNC development, we also found H3.3 to be required both tissue- and cell-autonomously for CNC specification. Shield-stage transplantation of wild-type CNC precursors into h3f3adb1092/db1092 homozygous mutant hosts fully rescued snai2 CNC expression, fli1a:GFP-positive ectomesenchyme, and the head skeleton (Figure 8a–8f). Moreover, mosaic injection of D123N-H3.3 blocked the CNC expression of a sox10:GFP transgene in a strictly cell-autonomous manner (Figure 8g–8j).

Although defective at early stages (11 hpf), the CNC expression of sox10 partially recovered by 16.5 hpf in h3f3adb1092 mutants (Figure 6m), consistent with multiple CNC derivatives being unaffected or only mildly reduced. In contrast, the expression of the ectomesenchyme markers dbx2a and twist1a was greatly reduced in h3f3adb1092 mutants at 16.5 hpf (Figure 6m, 6o; Figure S5). The loss of ectomesenchyme expression corresponded to regions of sox10-expressing CNC that were absent in h3f3adb1092 mutants. CNC cells that normally become ectomesenchyme likely fail to migrate from the neural tube and die, as we observed an increase in sox10-positive cells in the dorsal neural tube (Figure 6m) and elevated cell death in this same domain of 16 hpf h3f3adb1092 embryos (Figure 9d–9f). Importantly, increased cell death was not observed in the h3f3adb1092 neural folds at 12.5 hpf (Figure 9a–9c), indicating that the earlier loss of CNC gene expression was not due to cell death.

Discussion

It is becoming increasingly appreciated that changes in chromatin states are intimately tied to changes in cellular potential. During differentiation of many stem/progenitor cells, combinatorial histone marks associated with the promoters and enhancers of poised developmental genes are progressively resolved to active or inactive states in a lineage-specific manner [7–10]. In the germ line, rather than progressive enzymatic modifications of histone marks, the global replacement of existing histones with H3.3 and other variant histones is thought to contribute to a dramatic alteration of lineage
potential [17]. The unusual developmental history of ectomesenchyme may explain why it too is uniquely sensitive to defects in the incorporation of H3.3 replacement histones. CNC ectomesenchyme derives from the ectoderm yet expresses a gene repertoire and gives rise to derivatives (e.g. skeleton) in common with the mesoderm. One possibility then is that an H3.3-dependent histone replacement event in the NPB, which by its widespread nature is particularly sensitive to defective H3.3 incorporation, endows ectoderm-derived CNC cells with their exceptionally broad lineage potential, including the ability to generate mesoderm-like derivatives (Figure 10). The requirement for histone replacement (as opposed to enzymatic histone modifications) could reflect a need to overcome a particularly entrenched level of silencing during this fate transition, or alternatively to actively maintain mesoderm-like potential from earlier developmental stages [32]. In contrast, the progressive fate restriction of most other embryonic lineages may depend more on an incremental refinement of chromatin structure, such as the post-translational modification of histone tails and repositioning of existing nucleosomes, and thus be less sensitive to defective H3.3 incorporation.

Potential (17). The unusual developmental history of ectomesenchyme may explain why it too is uniquely sensitive to defects in the incorporation of H3.3 replacement histones. CNC ectomesenchyme derives from the ectoderm yet expresses a gene repertoire and gives rise to derivatives (e.g. skeleton) in common with the mesoderm. One possibility then is that an H3.3-dependent histone replacement event in the NPB, which by its widespread nature is particularly sensitive to defective H3.3 incorporation, endows ectoderm-derived CNC cells with their exceptionally broad lineage potential, including the ability to generate mesoderm-like derivatives (Figure 10). The requirement for histone replacement (as opposed to enzymatic histone modifications) could reflect a need to overcome a particularly entrenched level of silencing during this fate transition, or alternatively to actively maintain mesoderm-like potential from earlier developmental stages [32]. In contrast, the progressive fate restriction of most other embryonic lineages may depend more on an incremental refinement of chromatin structure, such as the post-translational modification of histone tails and repositioning of existing nucleosomes, and thus be less sensitive to defective H3.3 incorporation.

The open permissive chromatin structure at poised regulatory regions is characterized by low nucleosome occupancy and high H3.3 levels, although it remains unclear whether H3.3 simply fulfills a neutral replacement function in such areas of high turnover or has a facilitative destabilizing role [33,34]. An important question is which genes, and in particular which regulatory regions, are targets of histone replacement in the early NPB/CNC. Early CNC genes such as sox10 and foxd3, whose

Figure 6. H3.3 functions at the NPB–CNC transition. a–f, Expression of msxb, pax3a, zic2a, and tfap2a at 10 hpf and msxb and pax3a at 11 hpf is indistinguishable between wild types and both homozygous and heterozygous h3f3ad−/− mutants (mut) (n>10 for each). g–k, At 11 hpf, both homozygous and heterozygous h3f3ad−/− mutants have severe reductions in the expression of snai2 (6/4 mut; 0/4 wt), sox10 (10/12 mut; 0/6 wild-type), foxd3 (9/9 mut; 0/5 wt), tfap2a (7/7 mut; 0/4 wt), and sox9b (5/8 mut; 0/3 wt). l, sox10 expression is also lost in embryos injected with D123N (10/12) but not wild-type (0/16) h3f3ad mRNA. m, In both homozygous and heterozygous h3f3ad−/− embryos, sox10 expression partially recovers by 16.5 hpf yet is specifically reduced in presumptive CNC ectomesenchyme domains (arrows) (4/4 mut; 0/4 wt). An increase in sox10-positive cells is evident in the mutant dorsal neural tube (insert) between the sox10-positive otic placodes (arrowheads) which are unaffected in mutants. n, At 16.5 hpf, dlx2a expression in three streams of migrating ectomesenchyme is reduced in both homozygous and heterozygous h3f3ad−/− mutants (10/7 mut; 0/5 wt). o, The 16.5 hpf ectomesenchyme expression (arrows) of twist1a is reduced in both homozygous and heterozygous h3f3ad−/− mutants yet paraxial mesoderm expression is unaffected (white arrowheads) (8/8 mut; 0/5 wt). In all panels, homozygous h3f3ad−/− examples are shown. All images are dorsal views with anterior to the left. Scale bars: 250 μm.
expression is dramatically perturbed in the presence of mutant D123N H3.3, could be direct targets, although it is unclear why their activation would uniquely depend on H3.3. Alternatively or in parallel, the regulatory regions of a larger set of lineage-specific genes, in particular those associated with later ectomesenchyme development, could be primed by H3.3-dependent histone replacement during early CNC specification. Future chromatin immunoprecipitation studies, currently not feasible due to the rarity of CNC precursors in zebrafish embryos, will clearly be critical for determining which types of genes are direct targets of histone replacement during CNC specification.

Another unresolved question is how H3.3 incorporation is specifically targeted to CNC genes/enhancers. Several putative H3.3 chaperones have been identified, including Hira [35,36], Daxx [37], and Dek [38]. However, effective morpholino-mediated reduction of zebrafish hira, daxx, or dek gene products, either alone or in combination, failed to cause CNC-specific defects (Figure S6). Hira−/− and Dek−/− mouse mutants also do not display CNC-specific defects [39,40], whereas Daxx−/− embryos die around E8.5 before CNC can be extensively analyzed [41]. Whereas evidence for Hira, Daxx, or Dek mediating H3.3 incorporation in CNC is inconclusive, an intriguing alternative candidate is the chromatin remodeling complex CHD7-PBAF. Losses of CHD7-PBAF components disrupt early CNC specification in a manner similar to mutant D123N H3.3, with reduced expression of early CNC gene expression but not upstream NPB factors [11,12], and CHD7 genomic localization coincides with H3K4me1 marks which are particularly enriched in H3.3 [34,42]. It is less clear to what extent CHD7-PBAF, as well as the histone demethylase jmjD2A, share similar requirements with H3.3 for CNC lineage potential. As in h3f3adb1092 mutants, inhibition of CHD7 function in Xenopus laevis embryos disrupts craniofacial cartilage development, yet the effects on other NC lineages were not examined. In contrast, depletion of jmjD2A in avians affected development of the CNC-derived ganglia, a structure that is specifically spared in h3f3adb1092 mutants. Whether CHD7-PBAF and jmjD2A act together with H3.3 histone replacement at similar regulatory regions of early CNC genes, or whether these complexes have distinct roles in CNC specification and subsequent lineage diversification, will be fertile areas for future research.

h3f3adb1092 mutants display an initial delay in the expression of all CNC genes examined, which then translates into a more restricted loss of CNC-derived ectomesenchyme. How then does this specific loss of ectomesenchyme relate to the earlier delay in CNC specification? In one model, early-forming CNC cells encounter local environmental cues that promote ectomesenchymal fates [43], with a delay in CNC formation causing cells to miss such cues. Alternatively, the delay in CNC appearance, and its later inability to form the normal range of derivatives, may both result from defects in H3.3-dependent epigenetic remodeling during early CNC stages. Indeed, studies in zebrafish indicate that the future lineage potential of CNC may be determined at very early stages [25]. In addition, the cranial pigment lineage defects of h3f3adb1092 mutants indicate more general roles for H3.3 histone replacement in CNC lineage potential. In contrast to trunk NC [3], the existence of a multipotent precursor
for all CNC lineages has yet to be demonstrated in vivo. Evidence in avians suggests that the ectomesenchyme arises from a temporally and spatially distinct subpopulation of CNC from precursors of other derivatives [6,44], and lineage tracing studies in zebrafish show that CNC cells are largely unipotent at early stages of development (13 hpf) [5,45]. Hence, despite individual cultured avian CNC cells being able to generate all derivatives [2], the embryonic CNC may be heterogeneous from initial stages. H3.3 histone replacement could therefore be selectively required for ectomesenchyme and pigment cell potential, but not neuroglial potential, within a common multipotent CNC precursor, or alternatively within distinct subpopulations of ectomesenchyme and pigment cell precursors (Figure 10). In the future, techniques to more severely perturb H3.3 histone replacement should help reveal whether the apparent lack of CNC neuroglial and trunk NC defects in \(h3f3adb1092\) mutants reflects a fundamentally different mode of chromatin remodeling in the development of these NC populations, as well as other embryonic cell types, or merely a lower sensitivity to loss of H3.3 function.

**Materials and Methods**

**Ethics statement**

All animals were handled in strict accordance with good animal practice as defined by the relevant national and/or local animal welfare bodies, and all animal work was approved by the
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University of Southern California Institutional Animal Care and Use Committee.

Isolation and genotyping of the db1092 mutation

For the genetic screen, wild-type AB adult males were mutagenized with ethyl nitrosourea and crossed with fli1a:GFP females to create fli1a:GFP mutant carriers. Using early pressure to inhibit meiosis II, parthenogenic diploid F1 progeny [46] were created from these carriers and analyzed under a fluorescent dissecting microscope for cartilage and Alizarin Red for bone and teeth [48]. In situ immunohistochemistry, the primary antibody HuC (Molecular Probes) was used at 1:200, and embryos were processed as described [63].

With the exception of the experiments in Figure S1 and Figure S5, all embryos were genotyped after image acquisition to confirm the segregation of the observed phenotypes with the h3f3a<sup>db1092</sup> mutation. In Figure S1, reductions in the fli1a:GFP-positive ectomesenchyme (known to correlate precisely with the presence of the h3f3a<sup>db1092</sup> mutation in our other experiments) was used to identify mutant embryos. In Figure S5, the presence of dIx2a reductions (also known to correlate with h3f3a<sup>db1092</sup> genotype in our other experiments) was used to identify h3f3a<sup>db1092</sup> mutants.

Construction of expression vectors and mRNA injections

Constructs used to generate mRNA were created using a Gateway cloning system (Invitrogen) that has been modified for use in zebrafish [64]. h3f3a (wild-type and db1092) and H3.2 (cDNA zgc:158629) open reading frames (ORFs) were amplified from embryonic cDNA using primers with attB1 and attB2R 5’ extensions (underlined) for cloning into the pDONR-221 middle entry vector. We also incorporated a consensus Kozak sequence immediately upstream of the start ATG in the forward primer (bold/italic). Primers used include the following: h3f3a-<br>

| Vector | Primer Target | Sequence |
|--------|---------------|----------|
| h3f3a<sup>db1092</sup> | h3f3a-attB1 | GGGGACAAGTTTGTACAAAAAAGCAATGATCGATGGAAA |<br>
| h3f3a<sup>db1092</sup> | h3f3a-attB2R | GGGGACCACT-9TAATACGACTCACTATAGGACCAACAGGCTTAAGGAAGAAGAGGA, twist3a-attB1 |<br>
| h3f3a<sup>db1092</sup> | h3f3a-attB2R | TGCGCTCGAG-9TAATACGACTCACTATAGGCCTTTATCTCTATGGTAAAC |<br>
| h3f3a<sup>db1092</sup> | h3f3a-attB1 | GGGGACAAGTTTGTACAAAAAAGCAATGATCGATGGAAA |<br>
| h3f3a<sup>db1092</sup> | h3f3a-attB2R | GGGGACCACT-9TAATACGACTCACTATAGGACCAACAGGCTTAAGGAAGAAGAGGA, twist3a-attB1 |<br>
| h3f3a<sup>db1092</sup> | h3f3a-attB2R | TGCGCTCGAG-9TAATACGACTCACTATAGGCCTTTATCTCTATGGTAAAC |<br>
| h3f3a<sup>db1092</sup> | h3f3a-attB1 | GGGGACAAGTTTGTACAAAAAAGCAATGATCGATGGAAA |<br>
| h3f3a<sup>db1092</sup> | h3f3a-attB2R | GGGGACCACT-9TAATACGACTCACTATAGGACCAACAGGCTTAAGGAAGAAGAGGA, twist3a-attB1 |<br>
| h3f3a<sup>db1092</sup> | h3f3a-attB2R | TGCGCTCGAG-9TAATACGACTCACTATAGGCCTTTATCTCTATGGTAAAC |<br>

Figure 9. Cell death in h3f3a<sup>db1092</sup> embryos. a–c, Lysotracker Red staining marks similar amounts of dying cells in wild-type (n = 2), h3f3a<sup>db1092</sup> heterozygous (n = 5), and h3f3a<sup>db1092</sup> homozygous (n = 3) embryos at 12.5 hpf. The bright staining in the bottom of each panel is the yolk. d–f, At 16 hpf, increased Lysotracker Red staining (arrows) was evident in the dorsal neural tube of h3f3a<sup>db1092</sup> heterozygotes (2/2) and h3f3a<sup>db1092</sup> homozygotes (3/3) but not wild types (0/4). These dying cells were located in a similar position to where CNC forms in wild-type embryos. Scale bar = 50 μm.

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GGCTCCACCATGGCAAGAACCAAGCAGAC, H3.2-attB2R – GGGGACCCTTTGTAAGAAAGCTGGGTAG- CCTTTGGGTTTAAATCAGG. PCR products were cloned into the pDONR-221 vector. Fusion PCR [65] was used to incorporate the D123N mutation into H3.2. In the first step the pDONR-221:H3.2 construct was used as template for PCR with two sets of primer pairs (A and B; C and D), with overlapping primers B and C containing the required mutation (lowercase in primer sequences): AB amplicon: Primer A (upstream of pDONR-221 integration site) - CAAATTGATGAGCAATGCTTTTT, Primer B – TGGATGTtCTTGGGCATGAT; CD amplicon: Primer C – CATGCCCAAGaACATCCAG, Primer D (downstream of pDONR-221 integration site) – GAGCTGCCAGGAAACAGC- TA. In the second step these two PCR products were combined and used as template for PCR with the H3.2-attB1 and H3.2-attB2R pDONR-221 cloning primers above, followed by cloning into pDONR-221. The fusion PCR technique was also used to generate N-terminal mCherry fusions for h3f3a and H3.2 constructs. Primers were designed to amplify the mCherry ORF excluding the stop codon (primers A and B) and the h3f3a/H3.2 ORF (primers C and D), with overlapping primers B and C encoding the linker sequence GSRPVAT (used previously for YFP-H3.3 N-terminal fusions [66] ; linker sequence in lowercase below) and primers A and D having attB1 and attB2R 5’ extensions (underlined), respectively: mCherry-A - GGGGA- CAAGTtTTGTACAAAAAAGCAGGGCTCCACCATGGTGAG-

Figure 10. Model for the role of H3.3-dependent histone replacement during CNC development. At the early embryonic blastula stage, cells have a broad potential with cis-regulatory elements for developmental genes existing in a "poised" chromatin state. After gastrulation occurs to form the three major germ layers (ectoderm, mesoderm, and endoderm), genes associated with a particular germ layer are activated or maintained in a poised state, whereas genes for other layers are strongly repressed at the chromatin level. The cranial neural crest (CNC) is unusual in that it is derived from ectoderm yet can give rise to mesoderm-like derivatives such as skeleton. H3.3-dependent histone replacement could thus be required to remodel the enhancers of mesodermal genes needed for ectomesenchymal fates, with the distinctive role of H3.3 in CNC correlating with the need to derepress mesodermal enhancers that have been previously silenced in the ectoderm germ layer (1). Alternatively H3.3 incorporation could act to maintain mesoderm-like potential in the CNC ectoderm from an earlier time in development (2). It also remains unresolved the extent to which ectomesenchyme derivatives (e.g. head skeleton) and non-ectomesenchyme derivatives (e.g. pigment, glia, and neurons) derive from a common multipotent precursor. Hence, the cranial pigment and ectomesenchyme defects of h3f3a<sup>dbn1092</sup> mutants could arise from altered histone replacement in a common multipotent precursor, or alternatively from independent defects in different subsets of heterogeneous CNC with more limited potential.

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CAAGGGCGAGG, mCherry-B - tgtagccacagctgatcCTTG-TACAGCTTCGTCCATG, h3f3a-C - ggttagctgcttgtgtacaAT-GGGCCGTCATACAGCAGAC, h3f3a-D - k3f3a-attB2R (above), H3.2-C - ggttagctgcttgtgtacaATGGCCAGAACGAGA-C, H3.2-D - H3.2-attB2R (above). PCR with AB and CD primer pairs were performed using the appropriate plasmid template (pME-mCherry, pDONR-221:k3f3a or pDONR-221:H3.2) followed by gel purification of the products. In the second step these two PCR products were combined (mCherry product AB with product CD from k3f3a or H3.2) and used as template for PCR AD with the mCherry-A and k3f3a-attB2R/H3.2-attB2R cloning primers above, followed by cloning into pDONR-221. All pDONR-221 middle entry clones were checked by sequencing, before being assembled with a 5' entry vector (p5E-CMV/Sp6 containing the Sp6 RNA polymerase promoter) and a 3' entry vector (p5E-polyA containing the SV40 late polyA signal) within the pDestTo2pA2 destination vector using LR clonase II Plus (Invitrogen), as per manufacturer’s instructions. In order to generate capped mRNA from these constructs, plasmid minipreps were first linearized using an appropriate restriction enzyme(s) (pDONR-221:k3f3a – BglII, pDONR-221:H3.2 – XhoI, pDONR-221:mCherry-k3f3a – SpeI and XhoI, pDONR-221:mCherry-H3.2 SpeI and XhoI) and then used as template for in vitro transcription using the Sp6 Message Machine kit (Ambion). Zebrafish embryos were injected at the one-cell stage with capped mRNA from these constructs, plasmid minipreps (Invitrogen), as per manufacturer’s instructions. In order to generate capped mRNA from these constructs, plasmid minipreps were first linearized using an appropriate restriction enzyme(s) (pDONR-221:k3f3a – BglII, pDONR-221:H3.2 – XhoI, pDONR-221:mCherry-k3f3a – SpeI and XhoI, pDONR-221:mCherry-H3.2 SpeI and XhoI) and then used as template for in vitro transcription using the Sp6 Message Machine kit (Ambion). Zebrafish embryos were injected at the one-cell stage with capped mRNA at a concentration of 900 ng/µl (k3f3a, H3.2, mCherry-k3f3a and mCherry-H3.2) or 450 ng/µl (h3f3a mRNA rescue of k3f3aΔ61092 mutants). Alternatively, individual cells of 32-cell stage embryos were injected with mRNA encoding mCherry-h3f3a (900 ng/µl) to generate mosaic expression at later stages.

For the generation of constructs expressing FLAG-HA-tagged H3.3 in HEK 293T cells, the ORF of k3f3a was first amplified from wild-type and db1092 embryonic cDNA using forward and reverse primers with 5' extensions containing EcoRI and BamHI sites, respectively: 5′ – GGCAGCGGAATTCTAGATGCCGG-TACTAGGCAGAC, 3′ – GCCGCTAGTGGATCCATTAAGCC-TC TGTCCTCGTCGTGATG. Using these restriction sites the PCR product (see cloning protocol) was then cloned between EcoRI and BamHI sites downstream and in-frame with a Kozak-FLAG-HA sequence within a modified pIREsNeo vector (Clontech).

The pax3a:GFP line will be described in detail elsewhere. Briefly, the pax3a enhancer was identified by sequence conservation, then amplified from Fugu rubripes genomic DNA and cloned into the Tcd2 transgenesis vector pGreenE (unpublished) using BP clonase (Invitrogen). Constructs were injected with transposase mRNA into one-cell-stage embryos and germline transgenic founders were identified.

Nucleosome incorporation assay
HEK 293T cells were transfected with plasmids expressing FLAG-HA-tagged wild-type H3.3 (pIRES-FLAG-HA-H3.3), or mutant D123N H3.3 (pIRES-FLAG-HA-D123N-H3.3). To purify the whole cell nuclear extract, cultured cells were washed with PBS and lysed in IP lysis buffer (50 mM HEPES-KOH pH 7.6, 140 mM NaCl, 1 mM EDTA, and 10% Triton X-100) followed by western blotting using FLAG antibody (Sigma). To purify mononucleosomes, cell nuclei were digested with micrococcal nuclease (0.6 U, Sigma) as previously described [67] after expression of FLAG-tagged wild-type or D123N H3.3. Purification of mononucleosomes was confirmed by 2% agarose gel electrophoresis. For immunoprecipitation of FLAG-H3.3-containing mononucleosomes, M2-agarose beads (Sigma) were used followed by overnight incubation. Beads were washed five times with BC300 and mononucleosomes were electrophoretically analyzed on a 15% SDS polyacrylamide gel.

Co-immunoprecipitation
HEK 293T cells were transfected with pIRES-FLAG-HA-H3.3 or pIRES-FLAG-HA-D123N-H3.3. Cells were harvested 2 days after transfection and nuclear fraction was done as previously described [68]. 1 mg of nuclear fraction was incubated with 10 µl Flag Agarose (Sigma) for 4 hr at 4°C in nuclear extraction buffer (20 mM HEPES-KOH (pH 8.0), 0.6 M KCl, 1.3 mM MgCl2, 0.2 mM EDTA, 25% (vol/vol) glycerol, 1 mM DTT, 0.2 mM PMSF). This was followed by three washes in IP buffer and elution with 0.2 mg/ml Flag peptide (Sigma). Eluates were subjected to SDS-PAGE and western blot analysis and recombinant octomter was used as a control for western blot. Antibodies used for western blots were as follows: Flag (Sigma), H3 (Abcam), and H4 (Abcam).

Antisense morpholino oligonucleotides
The following antisense morpholino oligonucleotides were designed to block splicing of exon/intron boundaries in the transcripts of target zebrafish genes (designed by and ordered from Gene Tools, LLC): k3f3a exon 3/intron 3/4 – ACAATA-TAATCCTACCTGTAAGAGCG, h3f3a exon 1/intron 1/2 – CGGT-GTTTACGTCGTCTCACCATTGTG, daxx exon 3/intron 3/4 – ATTACTGTAAGACCGATACCCTGTC, daxx exon 3/intron 3/4 – ATTCGAAGAACCGCTTACAGCCC. One-cell stage embryos were injected with 3 nl of morpholino (MO) at 400 µM. RT-PCR was used to determine morpholino efficacy. Whole RNA was prepared from twenty 10 hpf embryos for each treatment (morpholino-injected and uninjected) using the RNAqeous -4PCR kit (Ambion), followed by cDNA synthesis using the RETROscript kit (Ambion). cDNA was used as template for PCR at 50 ng/µl using primers within exons flanking the targeted exon/intron boundary: k3f3a exon 3 forward – TCAAGCGTCTGTCGAGGAAA, k3f3a exon 4 reverse – TGCTTCTACAAATGAGCCTAGTG, h3f3a exon 1 forward – AAGCTCTTGAAGGCCGAGT- TG, h3f3a exon 2 reverse – TTGGCCACCTGTAGACACCT, daxx exon 3 forward – CACGTGCTGAAAGTGGACAA, daxx exon 4 reverse – TCCACAGACGTCGAGAAGGC, daxx exon 3 forward – CCAGAGTTGAGACAAAGGG, daxx exon 4 reverse – ATCCATAAAGACGCCGAGCA.

Scoring the severity of the h3f3aΔ61092 phenotype
Alcian Blue-stained 4 and 5 dpf larval head skeletons were scored blind for loss of cartilage elements using the following system: Grade 0, unaffected; Grade 1, truncation of dorsal hyosymplectic and palaquadrate cartilages; Grade 2, loss of hyosymplectic and palaquadrate (except pterygoid process); Meckel’s and ceratohyal cartilage reduced; Grade 3, loss of hyosymplectic and palaquadrate cartilages; Grade 4, loss of hyosymplectic, palaquadrate, and pterygoid; Grade 4, ethmoid plate truncated/lost, trabeculae remain; Grade 5, all CNC-derived cartilages absent.

NC transplantation
Unilateral tissue transplants were performed as described, with the non-recipient side acting as an internal control [69]. Briefly, one-cell-stage donor fli1a:GFP embryos were injected with Alexa 568 dye (Molecular Probes) and cells were moved to the CNC precursor domain of fli1a:GFP hosts at 6 hpf.

Lysotracker staining for cell death
Live embryos were manually removed from their chorions and incubated with Lysotracker Red (Invitrogen) diluted to 5 µM in embryo medium (EM). Embryos were then incubated for
30–45 minutes in darkness. After 4–5 rinses with EM, embryos were then fixed overnight with 4% PFA in PBS at 4°C. Embryos were then rinsed once with PBT before being dehydrated in a methanol/PBT series to remove background, and then stored at −20°C. Samples were subsequently rehydrated, followed by two washes in PBT, before being imaged by confocal microscopy.

**Equipment and settings**

Live embryos, whole-mount skeletal preparations, and in situ hybridization embryos were photographed with a Leica MZ16 stereomicroscope using a Canon PowerShot S80 digital camera and CameraWindow software. Fluorescent images were captured on a Zeiss LSM5 Pascal upright confocal microscope using excitation from Argon 488 and HeNe 543 lasers. Using Zeiss LSM software, a Z-stack of approximately 40 μm was captured for fli1a::GFP images and then digitally flattened into a single projection. For the images of fluorescently labeled histones, individual Z-sections from time-lapse movies were used. Next, all files were loaded into Adobe Photoshop CS2 and adjusted for levels and brightness and contrast. Care was taken to apply identical adjustments to images from the same set of experiments, and levels adjustments were limited to avoid removing information from the image.

**Supporting Information**

**Figure S1** NC derivatives in h3f3adb1092 mutants. a–d, Live views at 32 hpf (a, b) and 54 hpf (c, d) show reductions of head melanocytes (black) in h3f3adb1092/db1092 homozygotes (n = 22/28). The melanocytes of the eye and trunk were never affected. Cranial xanthophores (yellow), most clearly seen at the anterior limit of the head (arrowheads), were also largely unaffected, e, f. In confocal projections of fli1a::GFP embryos at 36 hpf, HuC antibody staining (red) labels neurons of the cranial ganglia – from left to right the trigeminal, anterior lateral line, auditory, and posterior lateral line – which are unaffected in h3f3adb1092/db1092 homozygotes (n = 8). In the merged images, fli1a::GFP (green) shows a reduction of CNC-derived ectomesenchyme in the mutant. Scale bars: a–d, 250 μm; e & f, 50 μm. (TIF)

**Figure S2** D123N H3.3 fails to localize to condensed chromosomes within NPB cells. Confocal images of 13 hpf embryos harboring the H2A.F/Z::GFP and NPB-specific pax3a::GFP transgenes and injected with wild-type or D123N versions of mCherry::H3.3 fusion proteins. a–d, GFP fluorescence of cells within the pax3a::GFP-labeled NPB domain. Whereas most cells are in interphase, the few cells in metaphase/anaphase (arrowheads in merged images: a–d’’) exhibit both GFP-labeled condensed chromosomes (H2A.F/Z::GFP) and more diffuse lower-level cytoplasmic GFP (pax3a::GFP). a–b’, Wild-type mCherry–H3.3 localizes within the condensed chromosomes of 14/14 metaphase/anaphase cells. c–d’, D123N mCherry–H3.3 fails to localize within condensed chromosomes and instead appears diffuse throughout pax3a::GFP-positive NPB cells after nuclear envelope breakdown. (0/14 cells exhibit chromosomal localization during metaphase/anaphase). Scale bar = 10 μm. (TIF)

**Figure S3** D123N H3.3 protein remains stable during mitosis. Time course of confocal images from H2A.F/Z::GFP embryos expressing D123N mCherry::H3.3 fusion protein, showing a cell (arrowhead in merged image a’) progressing through the stages of mitosis including metaphase (a’a/‘a” and b’/‘b”), anaphase (c’/c’/c” and d’/‘d”), telophase (e’/e’/e”) and the eventual establishment of two new daughter cells (arrowheads, b’/’b”). a–d, H2A.F/Z::GFP localizes to condensed chromosomes during both metaphase and anaphase. a–‘d’, In contrast, D123N mCherry–H3.3 fails to co-localize with H2A.F/Z::GFP and appears as a weak diffuse signal throughout the cell(s) after nuclear envelope breakdown. e’/e’/e”, H2A.F/Z::GFP and D123N mCherry–H3.3 subsequently become co-localized during the re-establishment of the nuclear membranes during telophase. f’/f”, Nuclear co-localization continues into interphase in both daughter cells. The rapid re-appearance of strong nuclear mCherry–H3.3 signal in telophase (16/16 cells over 2 embryos) confirms that the low-level diffuse D123N mCherry–H3.3 signal observed during metaphase/anaphase results from a failure to localize to condensed chromosomes rather than protein degradation. Scale bar = 10 μm. (TIF)

**Figure S4** Localization of wild-type mCherry–H3.3 within metaphase/anaphase cells of h3f3adb1092/db1092 embryos. Confocal images from wild-type and h3f3adb1092/db1092 homozygous embryos harboring the H2A.F/Z::GFP transgene and injected with mRNA encoding wild-type mCherry::H3.3 fusion protein. Merged images show that wild-type mH3.3 protein co-localizes with H2A.F/Z::GFP in the chromosomes of metaphase/anaphase cells (metaphase cells shown: arrowheads) in wild-type and h3f3adb1092/db1092 homozygotes (mutant, 21/21 cells in 3 embryos; wild-type, 15/15 cells in 3 embryos). Detailed analysis of fluorescence levels revealed no significant differences in the distribution of wild-type mH3.3 fluorescence between wild types and mutants. Scale bar = 10 μm. (TIF)

**Figure S5** h3f3adb1092 embryos lack CNC ectomesenchyme but have normal neural patterning. a, In wild-type embryos at 15.5 hpf, expression of dks2a (blue) marks a subset of the forebrain (FB) and three streams of migrating CNC-derived ectomesenchyme (1–3). In red, pax6a expression marks the hindbrain boundary (MHB) and egl2b expression marks rhombomeres 3 and 5 (R3 and R5) of the hindbrain. b, c, dks2a-positive ectomesenchyme is reduced (n = 18/18) in h3f3adb1092 heterozygous and embryos and completely lost (n = 3/9) or greatly reduced (n = 6/9) in h3f3adb1092 homozygotes at similar stages. Neural patterning was never affected in h3f3adb1092 heterozygotes and homozygotes. Scale bar = 100 μm. (TIF)

**Figure S6** Antisense morpholino targeting of H3.3 chaperones. a, Zebrafish have one predicted copy each of hira, daxx, and dke genes in their genomes. Antisense morpholino oligonucleotides were designed to inhibit splicing at specific exon/intron boundaries (green arrowheads) within hira (exon 1/intron 1–2), daxx (exon 3/intron 3–4), and dke (exon 3/intron 3–4) transcripts. Morpholinos were injected into one-cell-stage zebrafish embryos at 400 μM. b–d, Morpholino efficacy was demonstrated by PCR amplification between exons flanking targeted splice junctions from 10 hpf cDNA from 20 pooled embryos (position of primers shown as red arrows in a). Morpholino-treated samples exhibited a significant decrease in PCR product representing spliced transcript (b, hira, 96 bp; c, daxx, 143 bp; d, dke, 169 bp) and a concomitant increase in un-spliced PCR product (b, hira, 637 bp; d, dke, 247 bp) or an alternative spliced transcript (c, daxx, 169 bp: utilization of cryptic splice donor site 26 nucleotides into adjacent intron, predicted to result in frameshift and early termination). e–j, Wholemount in situ hybridization for sox10 at 11 hpf. Morpholinos against hira (l), daxx (h) and dke (j) have no effect on early sox10 expression within CNC cells when compared to un.injected controls (e, g, i) (n=9 for each). k–p, 5 dpf larval head skeletons stained with Alcian blue (cartilage) and Alizarin red (bone and chondrogenesis) and with antibody against sox10 (k–n). Scale bars: a–d, 50 μm; e–j, 100 μm; k–p, 1 mm. (TIF)
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Author Contributions

Conceived and designed the experiments: SGC HK JGC. Performed the experiments: SGC HK JGC. Analyzed the data: SGC JGC WA. Contributed reagents/materials/analysis tools: ATG DMM. Wrote the paper: SGC JGC.

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