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***cdon* and *boc* affect trunk neural crest cell migration through a non-cell autonomous
reduction of hedgehog signaling in zebrafish slow-twitch muscle**

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Abstract

The immunoglobulin superfamily members *cdon* and *boc* are transmembrane proteins implicated in regulating hedgehog signaling during vertebrate development. Recent work showing roles for these genes in axon guidance and neural crest cell migration further suggest that *cdon/boc* may play additional functions in regulating directed cell movements during development. Here we use novel and existing mutants to investigate a role for *cdon* and *boc* in zebrafish neural crest cell migration. We find that single *cdon* or *boc* mutant embryos exhibit normal neural crest phenotypes, but that neural crest migration is strikingly disrupted in double *cdon/boc* mutant embryos. We further show that this neural crest migration phenotype is associated with defects to the differentiation of slow-twitch muscle cells, and that this slow-twitch muscle phenotype is a consequence of reduced hedgehog signaling in mutant fish. While neural crest migratory ability is not affected in double mutant embryos, neural crest directionality is severely affected. These data suggest that neural crest migration defects are likely to be secondary to defects in slow-twitch muscle differentiation. Combined, our data add to a growing literature showing that *cdon* and *boc* act synergistically to promote hedgehog signaling during vertebrate development, and provide a foundation for using zebrafish to further study the function of these hedgehog receptor paralogs.

Introduction

During vertebrate development, neural crest cells (NCCs) migrate ventrally from the dorsal neural tube along conserved pathways in the embryo to contribute to numerous traits

such as the craniofacial skeleton, peripheral nervous system, and pigment (Theveneau and Mayor, 2012). Defects in NCC migration result in multiple human hereditary disorders (Tobin et al., 2008; Vega-Lopez et al., 2018), and modifications to NCC migration is associated with the evolution of morphological diversity among some vertebrate taxa (Powder et al., 2014). Further, diseases, such as metastatic melanoma, have been shown to progress through a reactivation of a NCC-like migratory genetic program (Diener and Sommer, 2021; Kaufman et al., 2016). Thus understanding the genetic regulation of NCC migration is fundamental to understanding the etiology to multiple human diseases as well as a major development event that occurs in every vertebrate embryo.

Current thinking stresses interactions among multiple mechanisms that when combined coordinate NCC migration along highly conserved routes in the embryo (Mayor and Etienne-Manneville, 2016). These include NCC intrinsic factors, such as genes regulating cell adhesion and actin cytoskeleton (Clay and Halloran, 2011, 2013, 2014; Williams et al., 2018), as well as cell extrinsic factors that affect the extracellular matrix (Perris and Perissinott, 2000; Szabo et al., 2016), tissue stiffness (Shellard and Mayor, 2021), and chemical gradients (Barriga et al., 2013; Olesnicky Killian et al., 2009; Shellard et al., 2018), which attract and funnel NCCs along conserved paths in the embryo. Mediating cell intrinsic and extrinsic regulation of NCC migration are cell-cell signaling interactions (Carmona-Fontaine et al., 2008; Mayor and Etienne-Manneville, 2016; Scarpa et al., 2015; Szabo et al., 2016; Theveneau and Mayor, 2012). These include the chemical gradients that attract and inhibit migrating NCCs, as well as the cell-cell adhesion molecules that adhere NCCs to each other and mediate many cell-cell interactions

(Carmona-Fontaine et al., 2008; Mayor and Etienne-Manneville, 2016; Scarpa et al., 2015; Szabo et al., 2016; Theveneau and Mayor, 2012; Vega-Lopez et al., 2017).

Recent work has implicated a role for the immunoglobulin superfamily member protein *cell adhesion associated, oncogene regulated (cdon)* as a cell surface protein regulating trunk NCC (tNCC) migration in zebrafish (Powell et al., 2015). Morpholino knockdown of *cdon* results in failed (tNCC) migration, with tNCCs mislocalizing N-cadherin and arresting migration at the body midline. *cdon* and its paralog *BOC, brother of cell adhesion associated, oncogene regulated (boc)*, are vertebrate members of a larger gene family with identifiable orthologs in other metazoan taxa that includes the drosophila paralogs *ihog* and *boi*. Recent work on both vertebrate *cdon/boc* and *Drosophila ihog/boi* has established a role for these transmembrane proteins in activating hedgehog signaling. *cdon* and *boc* act as co-receptors binding to hedgehog ligand and patched receptor in order to promote smoothened signaling within cells. Other work suggests that *cdon/boc* may commonly interact with additional signaling pathways including Wnt signaling and Nodal signaling (Hong et al., 2020; Jeong et al., 2017). Further, *cdon* has been shown to form a complex with N-cadherin/Cdc42/Bnip-2 in a hedgehog independent manner to regulate actin cytoskeletal dynamics during myogenic differentiation in cell culture (Kang et al., 2008; Lu and Krauss, 2010). *boc* has been shown to play a role in axon guidance, where it operates as a receptor for hedgehog ligand to regulate proper neuronal growth (Connor et al., 2005; Okada et al., 2006). Both *cdon/boc* and *ihog/boi* are often localized to the ends of long membrane extensions (e.g. cytonemes in *Drosophila*), suggesting that these receptors may help cells sense the wider external environment and communicate over long distances (Bilioni et al., 2013; Ferent et al., 2019; Hall et al., 2021). It is thus possible that these transmembrane

proteins are reiteratively used as cell surface proteins to integrate multiple signals during processes such as cell migration and axon guidance. While a role for *cdon* in tNCC migration has been suggested (Powell et al., 2015), the function of *cdon/boc* in regulating NCC movements is not fully understood.

In zebrafish, hedgehog signaling is currently thought to affect tNCC migration in a non-cell autonomous manner by regulating the differentiation of adaxial cell mesoderm (Honjo and Eisen, 2005). Adaxial cells form at the border of the notochord during the end of gastrulation and differentiate into slow-twitch muscle cells that migrate laterally through the somatic mesoderm (Elworthy et al., 2008; Wolff et al., 2003). A non-migratory population of adaxial cell derivatives, called muscle pioneers, remain at the body midline. In hedgehog mutant embryos, adaxial cells fail to differentiate into slow-twitch muscle/muscle pioneer derivatives (Elworthy et al., 2008; Roy et al., 2001; Yin et al., 2018). Through a mechanism not fully understood, but likely to depend on extracellular matrix deposition/modification (Banerjee et al., 2011; Banerjee et al., 2013; Guillon et al., 2016; Honjo and Eisen, 2005), this slow-twitch muscle differentiation is critical for normal NCC migration. Whether *cdon/boc* affect slow-twitch muscle differentiation, however, is unknown (Bergeron et al., 2011), and it is further possible that *cdon/boc* affect NCC development through NCC autonomous in addition to non-cell autonomous mechanisms.

Here we use novel CRISPR/Cas9 generated mutations and existing mutants to investigate the role of *cdon* and *boc* in tNCC migration and slow-twitch muscle differentiation. We show that *cdon* and *boc* are expressed by tNCCs at low levels and by the adaxial mesoderm at moderate levels. Loss of either *cdon* or *boc* individually has no observable effect on tNCC

migration nor slow-twitch muscle differentiation. However, embryos that are double mutant for both *cdon* and *boc* exhibit both tNCC migratory defects as well as loss of slow-twitch muscle mesoderm. We show that tNCC mobility is not impaired by loss of *cdon/boc* suggesting that the tNCC migration defect is a non-cell autonomous consequence of slow-twitch muscle differentiation. We further provide data suggesting that *cdon/boc* are modifying hedgehog signal reception during mesoderm differentiation. Combined, our data contribute to a growing literature showing that *cdon/boc* play a critical role in modulating hedgehog signaling across multiple tissues during embryogenesis.

Results

cdon/boc paralogs exhibit shared sequence conservation in extracellular functional domains

The transmembrane robo-related proteins, *cdon* and *boc*, share a similar genomic domain architecture that includes 4 immunoglobulin (IG) domains, 3 fibronectin type 3 repeat (FNIII) domains, and a cytosolic C-terminal tail (Figure 1A). A fifth IG domain is variable present in *cdon* orthologs of tetrapod taxa (grey in Figure 1A). The FNIII domains have been implicated in binding to hedgehog ligand and patched receptor (Izzi et al., 2011; Song et al., 2015), and some evidence suggests that the c-terminal region is needed for protein localization in the cell (Okumura et al., 2021).

As functional conservation is likely to be reflected by sequence conservation across evolutionary time, we investigated the molecular evolution of *cdon/boc* orthologs across vertebrate taxa. An alignment of *cdon* and *boc* amino acid sequences from 11 select jawed vertebrate taxa shows that extracellular domains exhibit high conservation across taxa and

paralogs, suggesting that these extracellular domains are critical for gene function (Figure 1A). In contrast, the C-terminal cytosolic tail exhibits relatively low sequence conservation (Kang et al., 2002) suggesting that cytosolic region is evolutionarily labile to different selection pressures.

Identifiable orthologs to *cdon* and *boc* are present in non-vertebrate bilaterian taxa. These include *Drosophila* paralogs *ihog* and *boi*, whose function in hedgehog signaling has been well characterized (Bilioni et al., 2013; Camp et al., 2010; Yao et al., 2006). However, the orthology relationships between *cdon/boc* and non-vertebrate orthologs such as *ihog* and *boi* are less well known. To explore the molecular evolution of this gene family we built a maximum likelihood tree (phymI) using 44 amino acid sequences from taxa representative for major bilaterian clades (Figure 1B). These data identify *cdon/boc* as shared vertebrate specific paralogs that emerge from a duplication event at either the base of the vertebrate phylogeny or along the gnathostome lineage following splitting from cyclostomes (hagfish and lamprey). Importantly, these data are consistent with a hypothesis that *cdon/boc* originated during one of the two vertebrate specific genome duplications (Dehal and Boore, 2005). Our analyses further suggest that an independent gene duplication event led to the origins of *boi* and *ihog* paralogs in drosophilids (Kang et al., 2002). Thus the co-orthologs *cdon/boc* and *boi/ihog* represent two independent duplication events (Figure 1B)(Allen et al., 2011; Izzì et al., 2011; Sanchez-Arrones et al., 2012), a finding that is concordant with gene trees made publicly available by the TreeFam consortium (Li et al., 2006; Ruan et al., 2008).

Generation of *cdon* and *boc* null alleles

To further study the function of *cdon* and *boc* during zebrafish development we turned to existing and novel mutations that are predicted to disrupt gene function (Figure 1C). Two previously published ENU generated *boc* alleles were used in this study to characterize the function of *boc* during zebrafish tNCC development. The *boc*/*uml* allele (Bergeron et al., 2011) results in a predicted early stop (hereafter *boc*^{*uml*}). The *boc*^{*co25*} allele (Kearns et al., 2021) results in an non-synonymous isoleucine to serine substitution in the third FNIII domain (Figure 1C). As *boc*^{*uml*} and *boc*^{*co25*} alleles do not complement each other (Kearns et al., 2021), we focus our analyses on *boc*^{*co25*} and note that we see similar results with *boc*^{*uml*}.

We used CRISPR/Cas9 to generate 2 novel mutations in *cdon*. Both alleles result in predicted early stops (Figure 1C). The *cdon*^{*co1019*} allele is a 229bp deletion that results in a predicted early stop leading to a truncated protein prior to conserved IG domains. The *cdon*^{*co1023*} allele is a 5bp deletion in the FNIII(2) domain that similarly results in a predicted early stop and a truncated protein prior to the transmembrane domain. We focus on the *cdon*^{*co1019*} allele and note that we see similar results with the *cdon*^{*co1023*} allele.

***cdon* and *boc* transcripts are expressed in tNCCs and slow-twitch muscle cells**

We next used multiplexed hybrid chain reaction *in situ* hybridization (HCR) in order to ask where *cdon* and *boc* transcripts are expressed, and whether transcripts are detectable in tNCCs and slow-twitch muscle progenitors. Unlike traditional enzymatic methods for *in situ* hybridization, HCR allows single molecule measurement of gene expression at cellular resolution, which enables identifying cells and cell types that express transcripts at relatively low levels (Kearns et al., 2021).

We found *cdon* and *boc* transcripts to be abundant in the dorsal central nervous system (CNS) of 24hpf-48hpf zebrafish embryos (Figure 2A-D), consistent with similar data from prior studies (Bergeron et al., 2011; Cardozo et al., 2014; Kearns et al., 2021). This pattern is established early in development. At 2 somites, *cdon* is expressed in the presumptive notochord, while both *cdon* and *boc* are both expressed along the border of the neural plate in cells that we suspect contribute to the dorsal neural tube at 24hpf (Figure 2A). Importantly, while *boc* transcripts in the neural tube form a broad dorsal-ventral gradient (Kearns et al., 2021), *cdon* transcripts are restricted to the dorsal-most roofplate cells and the ventral most floorplate cells of the neural tube by 24hpf. In this way, *cdon* creates a dorsal sub-domain of *boc* expression in the neural tube (Figure 2D). Floorplate cells also express hedgehog ligand, and thus unlike *boc*, *cdon* is expressed in both hedgehog ligand producing and non-producing cells within the CNS. This neural tube expression at 24hpf is broadly similar to expression patterns reported in mouse embryos (Izzi et al., 2011; Kang et al., 2002; Mulieri et al., 2002; MULIERI et al., 2000; Tenzen et al., 2006) suggesting that *cdon/boc* function in the CNS is likely to be conserved.

We next asked whether migrating tNCCs express *cdon* and *boc*. Using the tg(*sox10*:mRFP) transgene, we FAC sorted NCCs from the dissected trunks of 24hpf zebrafish embryos and performed bulk RNA sequencing. These data suggest that tNCCs do express *cdon* and *boc* at low levels (Figure 2E). We then asked if this expression is observable by HCR. Using the tg(*sox10*:mRFP) transgene to again label NCCs, we segmented out *cdon* and *boc* HCR puncta that overlap the *sox10* transgene. These data identify *cdon/boc* transcripts expressed by NCCs

at 24hpf (Figure 2F&G). While these data indicate that *cdon* and *boc* are expressed by tNCCs, we note that tNCCs are expressing *cdon/boc* at levels far lower than the surrounding tissues.

Of note is that mesoderm appears to express *cdon* and *boc* at relatively high levels (Figure 2), and prior studies had suggested that *boc* is expressed by slow-twitch muscle mesoderm at 24hpf (Bergeron et al., 2011). We thus, next sought to identify whether *cdon/boc* transcripts are in fact expressed by the developing slow-twitch muscle/adaxial cell mesoderm. To do this we used the tgBAC(*prdm1a*:eGFP) transgene to label adaxial cells and slow-twitch muscle mesoderm (Elworthy et al., 2008). At the 2 somite stage, adaxial cells are labeled with the *prdm1a*:eGFP transgene, but we did not observe significant expression of *cdon/boc* in these adaxial cells (Figure 2H). Instead, *cdon* and *boc* transcripts were largely restricted to the developing neural plate and notochord (see above). By the 10 somite stage, *cdon* and *boc* transcripts were observed throughout the paraxial mesoderm. Using the tg(*prdm1a*:eGFP) transgene to segment out HCR transcripts expressed by adaxial cells, we were able to confirm that *cdon* and *boc* are expressed by slow-twitch muscle progenitor cells (Figure 2I-J). At 24hpf, differentiating slow-twitch muscle cells continue to express both *cdon* and *boc* (Figure 2K-L). These data are consistent with bulk RNA-seq data from FAC-sorted trunk tgBAC(*prdm1a*:eGFP) positive cells at 24hpf (Figure 2E).

Taken together, our analyses indicate that *cdon* and *boc* are broadly expressed by mesodermal tissues that include adaxial cell derived slow-twitch muscle cells, as well as tNCCs at low levels, in addition to the developing neural tube.

Trunk neural crest migration and adaxial mesoderm development are affected in *cdon*/*boc* double mutants

We next asked whether *cdon* and *boc* mutations affect tNCC migration and slow-twitch muscle development by using the tg(*sox10*:mRFP) line to label migrating tNCCs and immunostaining of a slow-twitch muscle specific myosin (DSHB F59) to label adaxial mesoderm derivatives in 24 hpf embryos.

In wildtype embryos, tNCCs collectively migrate in streams down the middle of each body segment (Figure 3). We found that this tNCC migratory phenotype was largely normal in both single *cdon* and *boc* mutant embryos (Figure 3D-E). These data from homozygous mutant embryos are in contrast to those reported by morpholino knockdown of *cdon* where tNCC migratory streams fail to form (Powell et al., 2015). However, in double *cdon*^{co1019};*boc*^{co25} mutants, tNCCs did fail to form distinguishable streams, and tNCC migration arrested at the ventral neural tube/body midline (Figure 3F), a phenotypes that is similar to that observed in *cdon* morphant embryos (Powell et al., 2015).

Similar to tNCC migration, we found that slow-twitch muscle differentiation was largely normal in *cdon* and *boc* (Bergeron et al., 2011) single mutants. Only in double mutants did we observe defects in slow-muscle cell differentiation (Figure 3C-F). Loss of slow-twitch muscle cell differentiation was not complete, however, and posterior body segments tended to exhibit greater loss of slow-twitch muscle differentiation than anterior segments. In double mutant embryos, cells that did express slow-twitch muscle specific myosin (F59) appeared dysmorphic with loosely packed myosin bundles relative to wildtype embryos (e.g. compare myosin bundle width and packing in Figure 3).

Thus defects in tNCC migration and slow-twitch muscle cell differentiation are only observed in *cdon/boc* double mutants.

cdon and boc affect hedgehog signal transduction in slow-twitch muscle cells

Since *cdon* and *boc* have previously been implicated in hedgehog signaling (Allen et al., 2011; Zhang et al., 2006), we hypothesized that defects in both tNCC migration and slow-twitch muscle cell differentiation were due to loss of hedgehog signaling that is only apparent in the double mutants. To test this, we measured hedgehog signal reception by using the transgenic, tg(8xGliBS:mCherry-NLS-Odc1) reporter line (hereafter 8xGli:mCherry) that drives nuclear localized mCherry under the control of 8 tandem Gli binding sites (Mich et al., 2014). The mCherry fluorophore contains an Odc1 destabilization domain that facilitates proteasomal degradation to promote rapid fluorophore turnover within expressing cells.

At 24hpf, we observed mCherry positive nuclei in ventral neural tube cells and in slow-twitch muscle fiber nuclei (Figure 3G)(Kearns et al., 2021; Mich et al., 2014). We never observed mCherry positive nuclei in tNCCs (data not shown). In *cdon*^{co1019} and *boc*^{co25} mutants, the number and signal intensity of nuclei that are 8xGli:mCherry positive was lower as compared to wildtype siblings (Figure 3G-J). This decrease in signal was true for both cells in the neural tube and slow-twitch muscle cells. Importantly, 8xGli:mCherry signal intensity varied across genotypes, with *boc*^{co25} mutants displaying lower 8xGli:mCherry signal than *cdon*^{co1019} mutants, and double mutants exhibiting low to no signal than all other genotypes. These data suggest that *cdon*^{co1019} and *boc*^{co25} mutations are decreasing hedgehog signal reception in slow-twitch muscle cells, which is consistent with a hypothesis that perturbations to hedgehog signaling is

responsible for the slow-twitch muscle differentiation phenotype in *cdon* and *boc* mutant embryos (Barresi et al., 2000; Devoto et al., 1996).

Interestingly, the 8xGli:mCherry transgene reported substantively lower hedgehog signal reception in single *cdon*^{co1019} and *boc*^{co25} mutant embryos that exhibit normal slow-twitch muscle differentiation. These data suggest that slow-twitch muscle development is robust to moderate perturbations to hedgehog signal. If this hypothesis is correct, we reasoned that *cdon* and *boc* mutants should sensitize embryos to further pharmacological manipulation of smoothened signaling with cyclopamine. To test this, we in-crossed double heterozygous *cdon*^{co1019/+};*boc*^{co25/+} parents and treated embryos with 20uM cyclopamine from 50% epiboly to 24hpf. We then quantified the number of slow-twitch muscle fibers at 24hpf as a readout for hedgehog signaling.

While single *boc* and *cdon* mutants treated with ethanol as a controls had a similar number of slow-twitch muscle fibers as their wildtype ethanol treated siblings, *cdon* and *boc* mutants treated with 20uM cyclopamine had significantly fewer slow-twitch muscle fibers than their similarly cyclopamine treated wildtype siblings (Figure 4). This pattern was even more striking for the double mutant. While double mutants treated with ethanol have an average of approximately 5-6 slow-twitch muscle fibers per segment, double mutants lost all slow-twitch muscle fibers when treated with 20uM cyclopamine. Furthermore, we noted qualitative changes to slow-twitch muscle morphology in cyclopamine treated *cdon* and *boc* embryos. Mutants treated with 20uM cyclopamine often exhibited gaps or missing slow-twitch muscle fibers in body segments, and slow-twitch muscle fibers often appeared to have loosely packed myosin.

We interpret these results to indicate that *cdon* and *boc* are modifying hedgehog signal reception by slow-twitch muscles, and that loss of *cdon* or *boc* sensitize embryos to further perturbations of hedgehog signal.

Trunk neural crest cells lose directionality but not migratory ability in *cdon/boc* mutants

The arrested NCC migratory phenotype in the double mutants is striking and could result from either a loss of NCC migratory ability or a loss of directionality. To investigate this further we used the tg(*sox10*:mRFP) transgene to label migrating tNCCs and live imaged NCC migration through the trunk of the zebrafish embryo beginning at 22hpf (Figure 5). Migrating tNCCs were manually tracked in imageJ using the mTrackJ plugin (Meijering et al., 2012). As we only observed a tNCC migratory defect in the medial migrating streams, we limited our tracking of cells to NCCs migrating along the medial route. We found that tNCC mobility was not disrupted in *cdon*, *boc*, or double mutants as compared to wildtype controls. Across all genotypes, tNCCs were qualitatively migratory, and general measures of mobility (e.g. Max velocity etc.) in mutants were similar to that observed in wildtype controls (Figure 5D-G). In contrast, we observed a significant loss of directional NCC movement in double *cdon*^{co1019};*boc*^{co25} mutant embryos. In double mutants, tNCCs migrate to the body midline and then often move along the anterior-posterior axis (Figure 5A-C), which is in contrast to the directed ventral movement typical of wildtype embryos. This is seen quantitatively as lower migratory directionality and an increased proportion of movements in lateral directions (Figure 5B&H). Single *cdon* or *boc* mutants did not display migratory phenotypes that were different from that of wildtype controls. Taken together, we interpret these results to indicate that loss of *cdon* and *boc* affects

tNCC directionality and path-finding ability, but that *cdon* and *boc* are not required for tNCC mobility.

cdon and *boc* mutants exhibit hedgehog-like craniofacial phenotypes

While our data suggest that *cdon* and *boc* affect tNCC migration, we did not observe any defects in cranial NCC migration (data not shown). However we did observe severe defects to cranial cartilage morphology in *boc* and double mutants that are suggestive of other hedgehog mutants. Homozygous *cdon*^{co1019} and *cdon*^{co1023} mutants, however, do not display noticeable phenotypes (Figure 6). Further, we recovered homozygous *cdon* mutant adults that are fully viable. While these *cdon* alleles are apparently not deleterious to organismal viability, we note that when in combination with *boc* mutations these *cdon* alleles produce severe phenotypes (see below) supporting the prediction that these alleles disrupt normal *cdon* function based on the introduction of early stop codons.

In contrast to mutations in *cdon*, homozygous mutant *boc* embryos display striking craniofacial and curved body phenotypes that are characteristic of reduced hedgehog signaling (Bergeron et al., 2011). In the developing head, homozygous *boc*^{co25} and *boc*^{uml} mutants display neurocranial defects with fused trabecular cartilages (Figure 6A). Defects in the development of the neurocranial cartilages are classic midline phenotypes associated with mutations in hedgehog pathway genes (Bergeron et al., 2011; Eberhart et al., 2006; Wada et al., 2005). Interestingly, while mutations in *smoothed* or hedgehog ligand lead to complete loss of the anterior ethmoid plate and fusion of the posterior trabecular cartilages (Eberhart et al., 2006; Swartz et al., 2012; Wada et al., 2005), loss of *boc* only results in a relatively mild fusion of the

trabecular cartilages. Further, *boc* mutants have shortened or absent symplectic cartilages and a striking ventral oriented ceratohyal cartilage that attaches at an obtuse angle to the head (Figure 6). In the smoothened mutant (Swartz et al., 2012) and Dispatched 1 mutant (Schwend and Ahlgren, 2009) a rudimentary ceratohyal projects ventrally (Swartz et al., 2012), suggesting this mis-orientation of the ceratohyal is a canonical hedgehog phenotype. However, unlike in these other mutants where the ceratohyal is greatly hypomorphic and jaw joints are often fused, in *boc* mutants the ceratohyal is correctly formed and joints appear normal. To ask if this ceratohyal orientation defect was secondary to defects in soft tissue we stained craniofacial muscles with phalloidin and used the tg(*sox10*:mRFP) transgene to label NCC derived cartilages (Figure 6B). Craniofacial muscles appear normal, suggesting that the orientation of the ceratohyal in the *boc* mutant is due to cartilage element patterning and growth and not a secondary defect resulting from disrupted muscle differentiation and or attachment.

Double mutants homozygous for both *cdon*^{co1019} and *boc*^{co25} exhibit more extreme phenotypes consistent with a hypothesis that functional disruption of both *boc* and *cdon* leads to a more dramatic loss of hedgehog signal reception. In double mutants, the symplectic is absent, all craniofacial elements are hypomorphic, and neurocranial defects are more severe. Double mutants variably present mild to severe forms of cardiac edema and typically have more curved bodies than that observed in the *boc* mutants alone. We note that despite having more severe phenotypes, the ethmoid plate was present in all *cdon/boc* double mutant embryos observed, a curious observation given that the ethmoid plate is often lost in other hedgehog mutants. Studied *cdon* and *boc* mutations do not complement each other and *cdon*^{co1019}/*cdon*^{co1023};*boc*^{uml}/*boc*^{co25} embryos present phenotypes similar to homozygous

cdon^{co1019};*boc*^{co25} mutant embryos (Figure 6E). Taken together these data indicate that *cdon/boc* mutants display phenotypes similar to other characterized hedgehog mutants supporting the hypothesis that *cdon* and *boc* are modifying hedgehog signaling in the developing embryo head. That double *cdon/boc* mutants have more severe phenotypes and supports a hypothesis that *cdon/boc* paralogs have synergistic function in promoting hedgehog signal reception.

Discussion

Here we show that *cdon* and *boc* affect trunk NCC migration as well as differentiation of slow-twitch muscle cells. Despite some prior data suggesting that *cdon* may act cell autonomously to affect tNCC migration, we find that neither *cdon* nor *boc* mutations have a noticeable effect on NCC mobility. Rather, only in double *cdon/boc* mutants do we observe a noticeable NCC migratory defect that is associated with loss of slow-twitch muscle mesoderm. Prior research has shown that slow-twitch muscle differentiation requires hedgehog signaling (Elworthy et al., 2008), and our data show that this slow-twitch muscle phenotype is due to a reduction in hedgehog signaling within *cdon/boc* double mutants. Taken together we interpret our data to suggest that a tNCC migratory defect in *cdon/boc* double mutants is a consequence of defects in mesodermal differentiation (Banerjee et al., 2011; Banerjee et al., 2013; Honjo and Eisen, 2005).

Our data add to a growing literature showing that *cdon* and *boc* act synergistically during zebrafish development to modify hedgehog signal reception and regulate the development of multiple traits (Echevarria-Andino and Allen, 2020; Song et al., 2015; Zhang et

al., 2011). Interestingly we found that *cdon* mutations alone do not produce any noticeable phenotypes in zebrafish, and we are able to recover adult homozygous mutant zebrafish. This is contrast to mouse, where loss of CDON results in mild holoproccephaly (Allen et al., 2011; Allen et al., 2007; Echevarria-Andino and Allen, 2020; Zhang et al., 2011). While it is possible that our *cdon* mutations do not result in full loss of function, we find this possibility unlikely. Both *cdon* mutations lead predicted early stops prior to the transmembrane domain, which is similar to the *boc^{uml}* mutation that does lead to a severe loss of function phenotype. Further, our *cdon* mutants do impact *cdon* function as double *cdon/boc* mutants exhibit extreme phenotypes suggestive of loss of *cdon* function. As both mutations lead to an early stop prior to the transmembrane domain, production of any truncated *cdon* protein in these mutants should lead to an extracellular pool of *cdon* that would be more likely to result in a dominant negative phenotype than in a mild hypomorphic allele. However, we cannot fully rule out the possibility that a late start rescues *cdon* protein resulting in a hypomorphic but functional receptor. Unfortunately antibodies do not reliably recognize zebrafish *cdon* protein, and thus future research could investigate whether other alleles targeting the FNIII(3) domain might result in more severe phenotypes.

An alternative possibility, however, is that *cdon* and *boc*, as modifiers of hedgehog signal, interact with other hedgehog pathway members resulting in species (or fish/mouse line specific) phenotypic responses to *cdon* mutations. Thus in zebrafish other hedgehog pathway members may provide robustness against *cdon* mutations allowing generally normal phenotypic development. This hypothesis is supported by our findings that *cdon* and *boc* mutations both sensitize embryos to cyclopamine treatment. Thus presumably *cdon* mutations

also sensitize embryos to genetic perturbations such as loss of other hedgehog pathway members (or modifications to their gene expression). Intriguingly, we observed that *cdon* gene expression is often nested within *boc* expression suggesting that *cdon* and *boc* act together to modify hedgehog morphogen gradients. Kearns et al. 2021 found that *patched-2* (*ptch2*) expression in the dorsal neural tube is expanded in *boc*^{co25} mutant embryos, suggesting that loss of *boc* alone leads to more dorsal hedgehog reception (e.g. *ptch2* is a readout for hedgehog signaling). These data were interpreted to suggest that *boc* sequesters hedgehog ligand driving hedgehog signal reception to ventral tissues. Expanding on this hypothesis, we note that *cdon* transcripts are positioned even more distally in the neural tube from hedgehog ligand source than are *boc* transcripts. Thus together *boc* and *cdon* gradients may act as strong sequestering agents to limit hedgehog signal activation to proper tissues and inhibit ligand activity in dorsal neural tube cells.

Intriguingly, we and others note that *cdon* and *boc* are lowly expressed in a broad range of tissues including the mesoderm, NCCs among others. Further *cdon*, but not *boc*, transcripts are also co-expressed in hedgehog ligand producing cells, and some studies have suggested that *cdon* plays a role in presenting hedgehog ligand to responsive cells (Hall et al., 2021). Thus it is likely that *cdon* and *boc* are playing different roles in different tissues, and that cellular context may be critical for determining the biological function of these receptors.

Here we present an early investigation into the phenotypes of *cdon* and *boc* mutations in zebrafish as a foundation to further study the interact roles of these two paralogs. Future work dissecting how these genes operate in different cellular contexts will address the diverse roles of these genes in hedgehog pathway function.

Materials and Methods

Fish stocks, husbandry, and generation of mutants

Adult fish and embryos were maintained at the University of Colorado Anschutz Medical school according to standard zebrafish rearing protocols and guidelines for the rearing and maintenance of vertebrate animals. The *boc*^{co25} mutant was previously recovered from an ENU screen (Kearns et al., 2021). The *boc*^{uml} mutant was acquired from the Zebrafish International Resource Center (*boc*^{ty54z}) and was described by (Bergeron et al., 2011).

We used CRISPR/Cas9 to generate novel mutations in *cdon*. Two sets of guide RNAs (sgRNA) were designed to target either the 2nd and 3rd exons of *cdon* (ggcagUUGaggagcagagUa, UggUaggagccUgUgagagc, UgcUUCagaaUcgcUgagag) or to target the 11th exon (UcgccaUUGagaUUGgggg, cUccacgcgaaaUgcagUga). DNA oligos were annealed to a common backbone scaffold that contains a T7 RNA polymerase site, which was used to generate sgRNAs used for injections (Williams et al., 2018). For each sgRNA set, our final CRISPR injection solution consisted of sgRNAs diluted to 20ng/uL, 1ug Cas9 Protein (Invitrogen TrueCut V2), and 5ug Rhodamine Dextran in a total volume of 10uL. Approximated 2-4nl were injected into the cell of a 1cell stage embryo, and F0 injected embryos were reared to adulthood. We recovered a single allele for each sgRNA set. These include the *cdon*^{co1019} allele, which is a 229bp deletion that spans the second to third exon of *cdon*, removes the intronic sequence between these exons, and is predicted to result in an early stop. The *cdon*^{co1023} allele is a 5bp deletion resulting in a predicted early stop in the 11th exon that codes for the second FNIII

domain. F0 injected embryos were outcrossed for a minimum of two generations to minimize effects of off target cutting.

Zebrafish *boc* and *cdon* mutants were crossed in to existing transgenic reporter lines including the tg(*sox10*(7.2kb):mRFP)^{vu234} that labels NCCs with cytosolic mRFP (Kucenas et al., 2008), and the gli reporter tg(8xgli-Xla.Cryaa:NLS-d1mCherry) line that has 8 gli-binding sites driving a destabilized nuclear localized mCherry (Mich et al., 2014). The tgPAC(*prdm1a*:EGFP) line was used to label adaxial mesoderm for reference (Elworthy et al., 2008).

cdon/boc molecular conservation and gene tree

Amino acid coding sequences for select bilaterian taxa were downloaded from NCBI GenBank. Orthologous sequences were searched directly or identified through BLAST searches and manual curation. The final set of sequences was chosen to include taxonomic representatives for major bilaterian taxa for which high confidence sequences are available. Effort was made to identify two paralogs for each taxon. Amino acid sequences were aligned using MUSCLE (v3.8.31) with default parameters (Edgar, 2004). Amino acid conservation (Figure 1A) was calculated by pairwise comparisons of *cdon/boc* homologues from 11 taxa (human, mouse, chicken, *Anolis*, frog, bichir, Gar, sheepshead minnow, medaka, catfish, zebrafish) and plotted in R by smoothing conservation values across 20 amino acid windows. Substitution models were selected using ProtTest3 by AIC (Darriba et al., 2011). A maximum likelihood gene tree was built using phyml (v3.3) and a JTT+I+G+F model on the entire unfiltered alignment with 1000 bootstraps (Guindon and Gascuel, 2003). Nodes with less than 60% bootstrap support were

collapsed into polytomies. We recovered similar results from filtered alignments that removed poorly aligned regions and regions with gaps.

Immunohistochemistry, in situ hybridization, and stains

Adaxial mesoderm derivatives were stained by fluorescent immuno-histochemistry against a slow-twitch muscle specific myosin (anti-MYH1A, F59, DSHB) at a concentration of 1:20 as previously described (Elworthy et al., 2008; Nguyen-Chi et al., 2012). Hybrid Chain Reaction (HCR) *in situ* hybridizations were performed as previously described (Choi et al., 2018; Lencer et al., 2021). Custom HCR probes for zebrafish *cdon* and *boc* were acquired from Molecular Instruments and used at a concentration of 2pMol/500uL Hybridization Buffer. Phalloidin-488 and phalloidin-647 stains (Thermo Fisher) were used at a concentration of 1:1000 to label muscle tissue.

Live imaging and tracking of migrating cells

For live-imaging and tracking, neural crest cells were labelled using the tg(*sox10*:mRFP) line. Embryos were mounted in 0.4% low melt agarose and neural crest cells were imaged over the yolk extension every 5 minutes from ~22hpf to ~30hp on an Andor spinning disk confocal microscope at 28C. Neural crest cells were manually tracked along the xy axis from maximum projection images using the ImageJ mTrackJ plugin (Meijering et al., 2012). Drift was accounted for using the Correct_3D_Drift.py plugin as implemented in ImageJ (Parslow et al., 2014). Quantitative analysis of track mobility was performed in R. A small number of short tracks with fewer than 30 points (2.5hrs) were excluded. As we also noted that NCCs often stopped

migrating upon reaching a ventral position we further limited tracks to a maximum of 6 hours in order to capture cell migration. Limiting all tracks to the same time (e.g. number of points) produces similar results. Roseplots depict cell movements with a velocity greater than 0.3uM/min, which empirically determined as a cutoff for capturing cell movements while also excluding noise from drift and changes in cell shape. Using different cutoffs produced similar results. Linear models were built in R by including track length as a fixed effect, and embryo identity and date of imaging as random effects, with the packages lme4 (Bates et al., 2015), lmerTest (Kuznetsova et al., 2017), and emmeans. Images of tracks were produced in napari (Sofroniew et al., 2019).

Pharmacological manipulation

For pharmacological manipulation, embryos were manually dechorionated and treated with 20uM cyclopamine in ethanol (5uL/mL) from 50% epiboly to 24 hpf. Embryos that appeared developmentally delayed were excluded.

Imaging, Statistical Analysis, and biological replication

All imaging was performed on either a Leica Sp8 Confocal, Andor spinning disk confocal, or Olympus BX compound microscope. Image analysis and presentation was performed in ImageJ or napari. All statistical analyses were performed in R. Experiments were repeated at least 3 times and biological variation was accounted for either in models and through inclusion of biological replicates.

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Figure Legends

Figure 1. *cdon* and *boc* are transmembrane receptors and share conserved domain structure.

(A) *cdon/boc* proteins share a domain structure that includes 4-5 immunoglobulin (IG) domains, 3 fibronectin (FNIII) domains, a transmembrane (TM) region, and a cytosolic C-terminal tail. A fifth IG domain (*) in *cdon* is found in some tetrapod taxa and indicated in grey. Percent conservation (20 amino acid sliding window) is shown for an alignment of *cdon* and *boc* amino acid sequences from 11 tetrapod and ray-finned fish taxa. Note that the extracellular domains exhibit high sequence conservation, while the cytosolic region is less conserved. **(B)** Maximum likelihood tree (phymI) for curated *cdon* and *boc* amino acid sequences from multiple bilaterian taxa. Note independent duplications for Ihog/Boi in drosophilids and *cdon/boc* in vertebrates. Node support was calculated based on 1000 bootstraps, and nodes with less than 60% bootstrap support were collapsed. **(C)** Schematic of *cdon* and *boc* mutant alleles that are characterized in the current study. Three alleles result in a predicted early stop, while *boc*^{co25}

contains a single nucleotide substitution that results in a non-synonymous isoleucine to serine substitution.

Figure 2. *cdon* and *boc* are expressed by NCCs and adaxial mesoderm cells. (A-C) HCR shows *cdon* (magenta) and *boc* (green) expression in the developing neural tube of 2 somite (A), 24 hpf (B), and 48 hpf (C) embryos. Note *cdon/boc* expression at the border of the neural plate (A), and in the roofplate/dorsal neural tube cells at 24 and 48 hpf. Additionally, *cdon* is expressed in the ventral floorplate cells of the neural tube and notochord (B&C). Weak *cdon/boc* expression is observed throughout the mesoderm. Panels B&C are 20uM sections at the level of the yolk extension (YE) in the trunk. (D) Schematic summarizing expression of *cdon/boc* in the neural tube from panels B&C. Gradient of hedgehog ligand produced by notochord and floorplate cells is indicated at left in grey, while expression of *cdon/boc* are indicated by magenta and green respectively. (E) Bulk RNA-seq from FAC-sorted trunk NCCs (blue) and slow-twitch muscle cells (red) suggests that both tissues express *cdon/boc*. Left & middle panels are boxplots showing *cdon/boc* expression levels in 2 replicate samples of both cell types. Right panel shows histogram of gene expression levels for all samples for all genes in the dataset aligned with boxplots in left two panels. The location of genes that are markers for NCCs (*sox10*) or slow-twitch muscle (*prdm1a*, *smyh1*) are indicated by arrows. (F&G) HCR shows expression of *cdon/boc* in trunk NCCs. (F) whole mount lateral view of the trunk of a 24 hpf embryo showing expression of *cdon* (magenta), *boc* (green), and NCCs (grey) labeled by the tg(*sox10*:mRFP) transgene. HCR puncta that overlap the *sox10* transgene were segmented out to visualize NCC expression of *cdon* (F') or *boc* (F''). Segmented *cdon/boc* expression (*) are shown in yellow. (G)

Image from a 20uM cross section at the level of the yolk extension in the trunk shows NCCs expressing *cdon/boc* transcripts. *cdon/boc* HCR puncta overlapping the *tg(sox10:mRFP)* transgene (*) are shown in panels G' and G''. **(H-L)** Slow-twitch muscle cells express *cdon* and *boc*. The *tg(prdm1a:eGFP)* transgene labels adaxial cells at 2 somites **(H)**, and derivative slow-twitch muscle cells at later stages **(I-L)**. *cdon/boc* transcripts do not overlap the adaxial cells at 2 somites **(H)**, but do overlap the slow-twitch muscle cells at 10 somites **(I-J)** and 24 hpf **(K-L)**. Panel I shows 3D rendered of cross section from whole mount imaged embryos. HCR puncta overlapping the *tg(prdm1a:eGFP)* transgene are shown in yellow in panels I' and I'' to highlight slow-twitch muscle expression of *cdon/boc*. Panel J shows 3D rendered lateral view with HCR puncta overlapping the *prdm1a* transgene (yellow). **(K)** At 24hpf slow-twitch muscle cells (grey) continue to express *cdon/boc* transcripts. Panels K' and K'' show overlapping puncta. **(L)** 20uM cross section of the trunk of a 24hpf embryo shows *cdon/boc* puncta overlapping the *tg(prdm1a:eGFP)* transgene.

Figure 3. *cdon* and *boc* double mutants have NCC migration and slow-twitch muscle differentiation phenotypes and a reduction in hedgehog signal reception. **(A)** Schematic cross sections show NCC (red) and adaxial mesoderm (green) migration from approximately 10 somites to 20 somites. Adaxial mesoderm cells are specified adjacent to the notochord (nc) and migrate laterally to differentiate into slow-twitch muscle fibers. Concurrent with adaxial cell migration, NCCs migrate ventrally from dorsal neural tube (NT). **(B)** Left Panel shows 3D rendering of a 10uM cross section from the trunk of a 15 somite embryo shows medially positioned adaxial mesoderm (green) and ventrally migrating NCCs (red). NCCs are labeled with

the *tg(sox10:mRFP)* transgene, and adaxial mesoderm are labeled with the *tg(prdm1a:eGFP)* transgene. Right panels show a lateral view of a 3D rendering of the trunk of a 24hpf embryo shows slow-twitch muscle mesoderm (green) and migrating NCCs (red). Note the streams of migrating NCCs and laterally position slow-twitch muscle. **(C-F)** Maximum projection images show NCC streams (*tg(sox10:mRFP)*) and immunostaining for slow-twitch muscle specific myosin (F59) for 24hpf wildtype embryos. Note loss of both streams and slow-twitch muscle fibers in the double mutant. All images taken over the yolk extension in the trunk. **(G-J)** Maximum projection images the *tg(8xGli:mCherry)* transgene in 24hpf embryos. Images taken over the yolk extension. Note mCherry positive nuclei in the slow-twitch muscle mesoderm (yellow arrows) and ventral neural tube (yellow arrowheads). *8xGli:mCherry* signal progressively decreases in *cdon/boc* mutants reflecting lower hedgehog signal reception.

Figure 4. *cdon* and *boc* sensitize zebrafish embryos to a reduction in hedgehog signal by cyclopamine treatment. **(A)** Representative images show slow-twitch muscle fiber morphology in 24hpf embryos treated with either 20uM cyclopamine or an equivalent volume of ethanol as a control. Note *cdon* and *boc* mutants exhibit more severe phenotypes than their wildtype siblings when treated with cyclopamine as evidenced by gaps in segments (arrows). Also note complete loss of slow-twitch muscles in the double mutant treated with cyclopamine. **(B)** Quantification of average number of slow-twitch muscle fibers per segment shows that *cdon* and *boc* mutations are sensitizing embryos to cyclopamine treatment. Note that the number of slow-twitch muscle fibers in single *cdon* and *boc* mutants are similar to wildtype siblings when

treated with ethanol, but significantly lower when treated with 20uM cyclopamine. Significance values are from Tukey pairwise post-hoc tests: $<0.05 = *$, $<0.001 = ***$.

Figure 5. NCC directionality, but not migratory ability, is disrupted in the *cdon/boc* double mutants. (A) Maximum projection images show migrating NCCs (*tg(sox10:mRFP)*) in the trunks of representative wildtype, *cdon*, *boc*, and double mutant embryos. Migratory tracks from individual cells are shown by colored lines. Note that NCCs form horizontal tracks at the body midline in the double mutant. **(B)** Matched Roseplots (left panels) showing directional movement and line plots (right panel) showing tracks for NCCs in each genotype. For both panels, dorsal is up and anterior is to the left. Samples sizes reflect #tracks/#Embryos. Note that ventral directionality of NCC movement is lower in the double mutant as best seen by the rose plot. **(C)** NCC migration tracks (colored lines) are overlayed onto outlines of the body (grey dashed lines) for representative embryos. Note the vertical aligned tracks in the wildtype, *cdon*, and *boc* mutants that reflects ventral migration. Tracks form horizontal lines in the double mutant reflecting NCC migratory movements along the anterior-posterior axis. Body outlines were traced from the starting timepoint and do not reflect growth. Somite segments in the double mutant are wider and dysmorphic. **(D-H)** Boxplots show quantification of NCC migration for maximum velocity **(D)**, mean velocity **(E)**, track length **(F)**, track displacement **(G)**, and track straightness **(H)**. Note that the double *cdon/boc* mutants are the only condition that is significantly different from wildtype embryos in track straightness. Small grey points are individual track values, large colored points are means for individuals, boxplots reflect

variability across tracks. **(I)** Schematic of track straightness which is calculated as track displacement divided by track length.

Figure 6. *cdon* and *boc* are important for proper craniofacial development. (A) Representative images of 7dpf larvae show craniofacial cartilages in *boc*^{co25}, *cdon*^{co1019}, and double mutants as shown by alcian blue (cartilage, blue) and alizarin red (bone, red) stain. Note the medial trabecular elements of the neurocranium are disrupted in *boc* and double mutants (arrows). Also note a ventral projecting ceratohyal cartilage in the *boc* and double mutant (arrow head). All phenotypes are more severe in the double mutant which also has hypomorphic craniofacial cartilages and lacks the symplectic arm of the hyosymplectic cartilage. Scale bars are 20uM. ch = ceratohyal, ep = ethmoid plate, hs = hyosymplectic, mc = Meckel's cartilage, op = opercle, pq = palatoquadrate, pt = pterygoid process, sy = symplectic, tr = trabecula. **(B)** Representative maximum projection images of 7dpf larvae stained with phalloidin (green) to label muscles. Cartilages are labeled by the *tg(sox10:mRFP)* transgene that marks NCCs (magenta). Arrow head points to ventral oriented ceratohyal in *boc* and double mutants. Note that craniofacial muscles are normal across all genotypes suggesting that mis-orientation of the ceratohyal is unlikely to be a secondary consequence of disruption to soft-tissue morphology. **(C)** Quantification of ceratohyal angle shows that *boc*^{co25} and double mutants have significantly more ventral projecting ceratohyals. **(D)** Quantification of symplectic length showing that the length of the symplectic is shorter in *cdon/boc* mutants. Significance in panels B&C is Tukey post-hoc test relative to wildtype values. Sample sizes are wildtype = 6, *cdon* = 8, *boc* = 11,

622 Double = 6. **(E)** A $cdon^{co1019}/cdon^{co1023}; boc^{co25}/boc^{uml}$ embryo showing that different *cdon* and
623 *boc* alleles do not complement each other.

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Figure 1

Figure 2

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Figure 6