eLife

Reviewed Preprint
v2 • September 3, 2024
Revised by authors

Reviewed Preprint
v1 • January 9, 2024

Developmental Biology

Genetic requirement of *dact1/2* to regulate noncanonical Wnt signaling and *calpain 8* during embryonic convergent extension and craniofacial morphogenesis

Shannon H Carroll, Sogand Schafer, Kenta Kawasaki, Casey Tsimbal, Amélie M Julé, Shawn A Hallett, Edward Li, Eric C Liao

Center for Craniofacial Innovation, Children's Hospital of Philadelphia Research Institute, Children's Hospital of Philadelphia, USA • Division of Plastic and Reconstructive Surgery, Department of Surgery, Children's Hospital of Philadelphia, USA • Shriners Hospital for Children, Tampa, USA • Department of Biostatistics, Harvard T.H. Chan School of Public Health, Boston, USA

Abstract

Wnt signaling plays a crucial role in the early embryonic patterning and development, to regulate convergent extension during gastrulation and the establishment of the dorsal axis. Further, Wnt signaling is a crucial regulator of craniofacial morphogenesis. The adapter proteins Dact1 and Dact2 modulate the Wnt signaling pathway through binding to Disheveled. However, the distinct relative functions of Dact1 and Dact2 during embryogenesis remain unclear. We found that *dact1* and *dact2* genes have dynamic spatiotemporal expression domains that are reciprocal to one another and to *wnt11f2*, that suggest distinct functions during zebrafish embryogenesis. We found that both *dact1* and *dact2* contribute to axis extension, with compound mutants exhibiting a similar convergent extension defect and craniofacial phenotype to the *wnt11f2* mutant. Utilizing single-cell RNAseq and *gpc4* mutant that disrupts noncanonical Wnt signaling, we identified *dact1/2* specific roles during early development. Comparative whole transcriptome analysis between wildtype, *gpc4* and *dact1/2* mutants revealed a novel role for *dact1/2* in regulating the mRNA expression of the classical calpain *capn8*. Over-expression of *capn8* phenocopies *dact1/2* craniofacial dysmorphology. These results identify a previously unappreciated role of *capn8* and calcium-dependent proteolysis during embryogenesis. Taken together, our findings highlight the distinct and overlapping roles of *dact1* and *dact2* in embryonic craniofacial development, providing new insights into the multifaceted regulation of Wnt signaling.

eLife assessment

This **valuable** study confirms the roles of Dact1 and Dact2, two factors involved in Wnt signaling, during zebrafish gastrulation and demonstrates their genetic interactions with other Wnt components to modulate craniofacial morphologies. The limitation of the study is that it does not distinguish primary from secondary effects for each factor, precluding an unambiguous interpretation of their roles in craniofacial morphogenesis. The findings of a new potential target of dact1/2-mediated Wnt signaling are potentially of value; however, experimental evidence supporting their functional significance remains **incomplete** due to inconsistent results and the inherent limitations of the overexpression study.

https://doi.org/10.7554/eLife.91648.2.sa4

Introduction

Wnt signaling is a crucial regulator of embryogenesis through its regulation of body axis patterning, cell fate determination, cell migration, and cell proliferation (Logan and Nusse 2004, Steinhart and Angers 2018, Mehta, Hingole et al. 2021). Current mechanistic understanding of Wnt signaling during embryogenesis includes an extensive catalog of ligands, receptors, co-receptors, adaptors, and effector molecules (Clevers and Nusse 2012, Niehrs 2012, Loh, van Amerongen et al. 2016, Mehta, Hingole et al. 2021). The intricate spatiotemporal integration of Wnt signaling combinations is an important focus of developmental biology and tissue morphogenesis (Petersen and Reddien 2009, Clevers and Nusse 2012, Loh, van Amerongen et al. 2016, Wiese, Nusse et al. 2018). Disruptions of Wnt signaling-associated genes lead to several congenital malformations which often affect multiple organ systems given their pleotropic developmental functions (Hashimoto, Morita et al. 2014, Shi 2022). Craniofacial anomalies are among the most common structural congenital malformations and genes in the Wnt signaling pathway are frequently implicated (Ji, Hao et al. 2019, Reynolds, Kumari et al. 2019, Huybrechts, Mortier et al. 2020). The roles of certain Wnt signaling components may change in different temporal and spatial contexts, requiring detailed developmental and genetic analyses.

Genetic approaches in zebrafish have identified a number of key Wnt regulators of early development, with gastrulation and craniofacial phenotypes (Brand, Heisenberg et al. 1996, Hammerschmidt, Pelegri et al. 1996, Heisenberg, Brand et al. 1996, Piotrowski, Schilling et al. 1996, Solnica-Krezel, Stemple et al. 1996, Schilling and Le Pabic 2009). The *silberblick (slb)* mutant, later identified as a *wnt11f2* mutant allele, exhibited gastrulation and midline craniofacial phenotypes that encompassed aspects of multiple mutant classes (Heisenberg, Brand et al. 1996). During early segmentation in the somite stage, the *wnt11f2* mutant developed a shortened anterior-posterior axis and partially fused eyes (Heisenberg, Brand et al. 1996). Subsequently, as the cranial prominences converge and the ethmoid plate (EP) formed, instead of a fan-shaped structure observed in wildtype embryos, the *wnt11f2* mutant formed a rod-like EP with significant deficiency of the medio-lateral dimension (Heisenberg, Brand et al. 1996, Heisenberg and Nusslein-Volhard 1997). Another mutant kny*pek (kny)*, identified as having a nonsense mutation in *gpc4*, an extracellular Wnt co-receptor, was identified as a gastrulation mutant that also exhibited a shortened body axis due to a defect in embryonic convergent extension (CE) (Solnica-Krezel, Stemple et al. 1996). In contrast to the *slb*/*wnt11f2 mutant*, the *gpc4* mutant formed an EP that is wider in the medio-lateral dimension than the wildtype, in the opposite end of the EP phenotypic spectrum compared to *wnt11f2* (Topczewski, Sepich et al. 2001, Rochard, Monica et al. 2016). These observations beg the question how defects in early

patterning and CE of the embryo may be associated with later craniofacial morphogenesis. The observation that *wnt11f2* and *gpc4* mutant share similar CE dysfunction and axis extension phenotypes but contrasting craniofacial morphologies was previously pondered (Heisenberg and Nusslein-Volhard 1997). This observation supports a hypothesis that CE mechanisms regulated by these Wnt pathway genes are specific to the temporal and spatial contexts during embryogenesis. As such, there may be Wnt signaling modifiers that act in specific temporal windows during development.

Dact (aka Frodo, Dapper) are scaffolding proteins that were identified to regulate Dishevelled (Dvl)-mediated Wnt signaling, both positively and negatively (Cheyette, Waxman et al. 2002, Gloy, Hikasa et al. 2002, Waxman, Hocking et al. 2004, Gao, Wen et al. 2008, Wen, Chiang et al. 2010, Ma, Liu et al. 2015, Lee, Cheong et al. 2018). Dact proteins bind directly to Dvl (Gloy, Hikasa et al. 2002, Brott and Sokol 2005, Lee, Cheong et al. 2018) and have been found to interact with and inhibit members of TGFβ and Nodal signaling pathways (Zhang, Zhou et al. 2004, Su, Zhang et al. 2007, Meng, Cheng et al. 2008, Kivimae, Yang et al. 2011). In chick and Xenopus, *Dact2* and *Dact1* are expressed (respectively) in the neural folds during neural crest delamination and these were found to be important in epithelial-mesenchymal transition (EMT), Wnt, and Tgfβ signaling (Hikasa and Sokol 2004, Schubert, Sobreira et al. 2014, Rabadan, Herrera et al. 2016). In mouse embryos, Dact1 expression was reported predominantly in mesodermal tissues as well as ectodermal-derived tissues (Hunter, Hikasa et al. 2006) and ablation of Dact1 resulted in defective EMT and morphogenesis at the primitive streak and subsequent posterior defects (Wen, Chiang et al. 2010). Mouse embryonic Dact2 expression has been described in the oral epithelium (Li, Florez et al. 2013) and ablation of *Dact2* causes increased cell proliferation (Li, Florez et al. 2013) and re-epithelialization in mice (Meng, Cheng et al. 2008) as well as zebrafish (Kim, Kim et al. 2020).

Previous experiments using morpholinos to disrupt *dact1* and *dact2* in zebrafish found *dact1* morphants to be slightly smaller and to develop a normal body. In contrast, *dact2* morphants were found to phenocopy described zebrafish gastrulation mutants, with impaired CE, shortened body axis, and medially displaced eyes. Importantly, prior work using morpholino-mediated gene disruption of *dact1* and *dact2* did not examine craniofacial morphogenesis except to analyze *dact1* and *dact2* morphants head and eye shapes from light microscopy (Waxman, Hocking et al. 2004). These experiments were carried out at a time when morpholino was the accessible tool of gene disruption (Nasevicius and Ekker 2000, Corey and Abrams 2001, Heasman 2002). Since CRISPR/Cas9 targeted gene mutagenesis became popularized, many reports of germline mutant phenotypes being discrepant from prior morpholino studies warranted revisiting many of the prior work (Kok, Shin et al. 2015) and careful interpretation given the caveats of each technology (Morcos, Vincent et al. 2015, Rossi, Kontarakis et al. 2015). More recently, a zebrafish CRISPR/Cas9 genetic *dact2* mutant has been generated and studied, but unlike in the *dact2* morphant, no developmental phenotypes were described (Kim, Kim et al. 2020).

Here we investigated the genetic requirement of *dact1* and *dact2* during embryogenesis and craniofacial development, using germline mutant alleles generated by CRISPR-targeted mutagenesis. We found an early developmental role for *dact1* and *dact2* during gastrulation and body axis elongation. We also characterized the resulting craniofacial defect in the *dact1/2* compound mutants. Differential single-cell RNA sequencing between wildtype, *dact1/2,* and *gpc4* mutants revealed distinct transcriptome profiles and the discovery of *calpain 8* (*capn8)* calcium-dependent protease to be ectopically expressed in the *dact1/2* mutants and functions to modify Wnt signaling and craniofacial morphogenesis.

Results

dact1 and *dact2* have distinct expression patterns throughout embryogenesis

To determine the spatiotemporal gene expression of *dact1* and *dact2* during embryogenesis we performed wholemount RNA *in situ* hybridization (ISH) across key time points (**Fig. 1**). Given that the described craniofacial phenotypes of the *dact2* morphant and the *wnt11f2* mutant are similar (Heisenberg and Nusslein-Volhard 1997, Waxman, Hocking et al. 2004), we also performed *wnt11f2* ISH to compare to *dact1* and *dact2* expression patterns.

During gastrulation at 8 hours post-fertilization (hpf; 75% epiboly), some regions of *dact1* and *dact2* gene expression were shared and some areas are distinct to each *dact* gene (**Fig. 1A**). Further, *dact* gene expression was distinct from *wnt11f2* in that *wnt11f2* expression was not detected in the presumptive dorsal mesoderm. Transcripts of *dact1*, *dact2*, and *wntllf2* were all detected in the blastoderm margin, as previously described (Gillhouse, Wagner Nyholm et al. 2004). Transcripts of *dact2*, and to a lesser extent *dact1*, were also detected in the prechordal plate and chordamesoderm (**Fig. 1A**). Additionally, *dact2* gene expression was concentrated in the shield and presumptive organizer or Nieuwkoop center along with *wnt11f2*. This finding is consistent with previously described expression patterns in zebrafish and supports a role for *dact1 and dact2* in mesoderm induction and *dact2* in embryo dorsalization (Thisse 2001, Gillhouse, Wagner Nyholm et al. 2004, Muyskens and Kimmel 2007, Oteiza, Koppen et al. 2010). At the end of gastrulation and during somitogenesis the differences in the domains of *dact1* and *dact2* gene expressions became more distinct (**Fig. 1B,C**). At tailbud stage, *dact1* transcripts were detected in the trunk and the posterior paraxial mesoderm, whereas *dact2* transcripts were detected in the anterior neural plate, notochord, and tailbud, similar to *wnt11f2* gene expression. However, *dact2* was unique in that its expression demarcated the anterior border of the neural plate. As *dact2* morphants exhibited a craniofacial defect with medially displaced eyes and midfacial hypoplasia (Waxman, Hocking et al. 2004), we examined *dact1* and *dact2* expression in the orofacial tissues. At 24 hpf we found some overlap but predominantly distinct expression patterns of *dact1* and *dact2* with *dact1* being more highly expressed in the pharyngeal arches and *dact2* being expressed in the midbrain/hindbrain boundary. Both *dact1* and *dact2* appeared to be expressed in the developing oral cavity. At 48 hpf *dact1* expression is consistent with expression in the developing craniofacial cartilage elements, while *dact2* expression appears within the developing mouth. The distinct cellular expression profiles of *dact1* and *dact2* were more clear in histological sections through the craniofacial region at 72 hpf. Utilizing RNAscope *in situ* hybridization, we found that *dact2* and the epithelial gene *irf6* were co-expressed in the surface and oral epithelium that surround the cartilaginous structures (**Fig. 1D**). This is in contrast to *dact1* which was expressed in the developing cartilage of the anterior neurocranium/EP and palatoquadrate of the zebrafish larvae (**Fig. 1D**).

We examined the differences in expression pattern between *dact1* and *dact2* using Daniocell single-cell sequencing data (Farrell, Wang et al. 2018) (**Fig. S1**). During embryogenesis, *dact2* was more highly expressed in anterior structures including cephalic mesoderm and neural ectoderm while *dact1* was more highly expressed in mesenchyme and muscle (**Fig. S1**). We also utilized this tool to compare *dact1* and *dact2* expression to *wnt11f2* and *gpc4* expression, known noncanonical Wnt pathway members with craniofacial phenotypes (Heisenberg, Brand et al. 1996, Solnica-Krezel, Stemple et al. 1996, Heisenberg and Nusslein-Volhard 1997). In general, we found *dact1* spatiotemporal gene expression to be more similar to *gpc4* while *dact2* gene expression was more similar to *wnt11f2*. These results of shared but also distinct domains of spatiotemporal gene expression of *dact1* and *dact2* suggest that the *dact* paralogs may have some overlapping developmental functions while other roles are paralog specific.

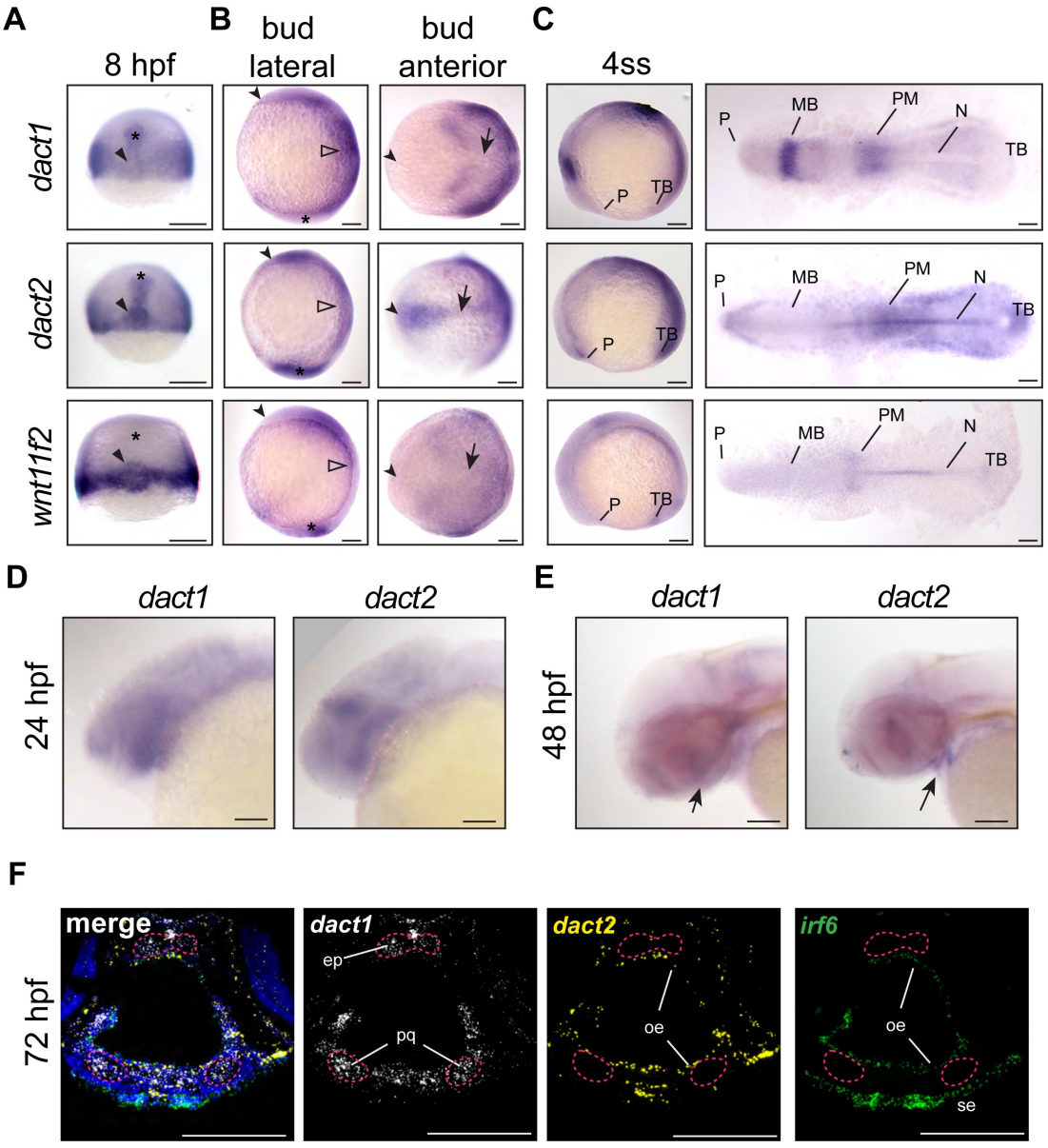


Figure 1.

Unique and shared *dact1* and *dact2* gene expression domains during zebrafish development. **(A-C)** Representative images of wholemount *in situ* hybridization showing *dact1*, *dact2*, and *wnt11f2* gene expression patterns. **A)** At 8 hpf, *dact2* and *wnt11f2* are highly expressed in the dorsal margin and presumptive Nieuwkoop center of the gastrulating embryo, with *dact1* being weakly detected (arrowhead). In contrast to *wnt11f2*, *dact1* and *dact2* are expressed in the presumptive dorsal mesoderm (asterisk). **B)** Lateral (anterior to the left of page) and anterior (dorsal side toward top of page) views of bud-stage embryos. *dact2* and *wnt11f2* transcripts are detected in the anterior neural plate (arrowhead) and tailbud (asterisk) while *dact2* is additionally expressed in the axial mesoderm (arrow). *dact1* gene expression is concentrated to the paraxial mesoderm (open arrowhead). **C)** Lateral and flat-mount views of 4 ss embryos. *dact2* is expressed in the anterior neural plate and polster (P), notochord (N), paraxial and presomitic mesoderm (PM) and tailbud (TB). *wnt11f2* is also expressed in these cells (Thisse, Pflumio et al. 2001). In contrast, *dact1* is expressed in the midbrain (MP) and the paraxial and presomitic mesoderm. (**D-E)** Representative lateral (anterior to left of page) images of wholemount *in situ* hybridization showing *dact1* and *dact2* expression patterns. **D)** At 24 hpf expression is detected in the developing head. **E)** At 48 hpf expression is detected in the developing craniofacial structures (arrow). **F)** Representative images of RNAscope *in situ* hybridization analysis of *dact1* (white) and *dact2* (yellow) and *irf6* (green) expression in transverse section of 72 hpf embryos. *dact1* is expressed in the ethmoid plate (ep) and palatoquadrate (pq) orofacial cartilage, while *dact2* is expressed in the oral epithelium (oe). The epithelial marker *irf6* is expressed in the oe and surface epithelium (se). Dapi (blue). Scale bar: 100 μm.

dact1 and *dact2* contribute to axis extension and *dact1/2* compound mutants exhibit a convergent extension defect

dact1 and *dact2* are known to interact with *disheveled* and regulate non-canonical Wnt signaling (Gloy, Hikasa et al. 2002, Waxman, Hocking et al. 2004, Gao, Wen et al. 2008, Wen, Chiang et al. 2010, Ma, Liu et al. 2015, Lee, Cheong et al. 2018) and we have previously described the craniofacial anomalies of several zebrafish Wnt mutants (Dougherty, Kamel et al. 2012, Kamel, Hoyos et al. 2013, Rochard, Monica et al. 2016, Ling, Rochard et al. 2017, Alhazmi, Carroll et al. 2021). Previous work investigated the effect of *dact1* and *dact2* disruption during zebrafish embryogenesis using morpholinos and reported morphant phenotypes in embryonic axis extension and eye fusion (Waxman, Hocking et al. 2004). However, the limitations associated with morpholino-induced gene disruption (Kok, Shin et al. 2015, Morcos, Vincent et al. 2015, Rossi, Kontarakis et al. 2015) and the fact that craniofacial morphogenesis was not detailed for the *dact1* and *dact2* morphants, warranted the generation of mutant germline alleles (**Fig. S2**). We created a *dact1* mutant allele (22 bp deletion, hereafter *dact1-/-*) and a *dact2* mutant allele (7 bp deletion, hereafter *dact2-/-*), both resulting in a premature stop codon and presumed protein truncation (**Fig. S2A,B**). Gene expression of *dact1* and *dact2* was measured in pooled *dact1-/-*, *dact2-/-*, and *dact1/2-/-* embryos (**Fig. S2C**). We found a decrease in *dact1* mRNA and no change in *dact2* mRNA levels in the respective CRISPR mutants. We hypothesize that *dact2* mRNA levels are maintained in the *dact2-/-* mutant due to the relative 3' position of the deletion. In the *dact2-/-* embryos we found a slight increase in *dact1* mRNA levels, suggesting a possible compensatory effect of dact2 disruption. The specificity of the gene disruption was demonstrated by phenotypic rescue of the rod-like EP with the injection of *dact1* or *dact2* mRNA. Injection of *dact1* mRNA or *dact2* mRNA or in combination decreased the percentage of rod-like EP phenotype from near the expected 25% (35% actual) to 2-7% (**Fig. S2D,E**).

Analysis of compound *dact1* and *dact2* heterozygote and homozygote alleles during late gastrulation and early segmentation time points identified embryonic axis extension anomalies (**Fig. 2A,B**). *dact1-/-* or *dact2-/-* homozygotes develop to be phenotypically normal and viable. However, at 12 hpf, *dact2-/-* single mutants have a significantly shorter body axis relative to wildtype. In contrast, body length shortening phenotype was not observed in *dact1-/-* homozygotes. Compound heterozygotes of *dact1+/-*; *dact2+/-* also developed normally but exhibited shorter body axis relative to wildtype. The most significant axis shortening occurred in *dact1-/-; dact2-/-* double homozygotes with a less severe truncation phenotype in the compound heterozygote *dact1+/-; dact2-/-* (**Fig. 2A,B**). Interestingly, these changes in body axis extension do not preclude the compound heterozygous larvae from reaching adulthood, except in the *dact1-/-; dact2-/-* double homozygotes which did not survive from larval to juvenile stages.

Body axis truncation has been attributed to impaired CE during gastrulation (Tada and Heisenberg 2012). To delineate CE hallmarks in the *dact1-/-;dact2-/-*mutants, we performed wholemount RNA ISH detecting genes that are expressed in key domains along the body axis. At bud stage, *dact1-/-; dact2-/-* embryos demonstrate bifurcated expression of *pax2a* and decreased anterior extension of *gsc* expression, suggesting impaired midline convergence and anterior extension of the mesoderm (**Fig. 2C**). At the 1-2 somite stage, *zic1*, *pax2a* and *tbx6* are expressed in neural plate, prospective midbrain and the tailbud, respectively, in both the wildtype and *dact1-/-; dact2-/-* embryos. However, the spacing of these genes clearly revealed the shortening of the anteroposterior body axis in the *dact1-/-; dact2-/-* embryos. Midline convergence is decreased and the anterior border of the neural plate (marked by *zic1* expression) was narrower in the *dact1-/-; dact2-/-* embryos (**Fig. 2D**). At the 10-somite stage (ss), *dact1-/-; dact2-/-* embryos demonstrated decreased spacing between *ctslb1* and *pax2a* gene expression, suggesting impaired lengthening of the anterior portion of the embryo. Detection of muscle marker *myo1d* in the *dact1-/-; dact2-/-* embryos delineated impaired posterior lengthening as well as reduced somitogenesis, evidenced by the decreased number of somites (**Fig. 2E**). These data point to impaired CE of the mesoderm

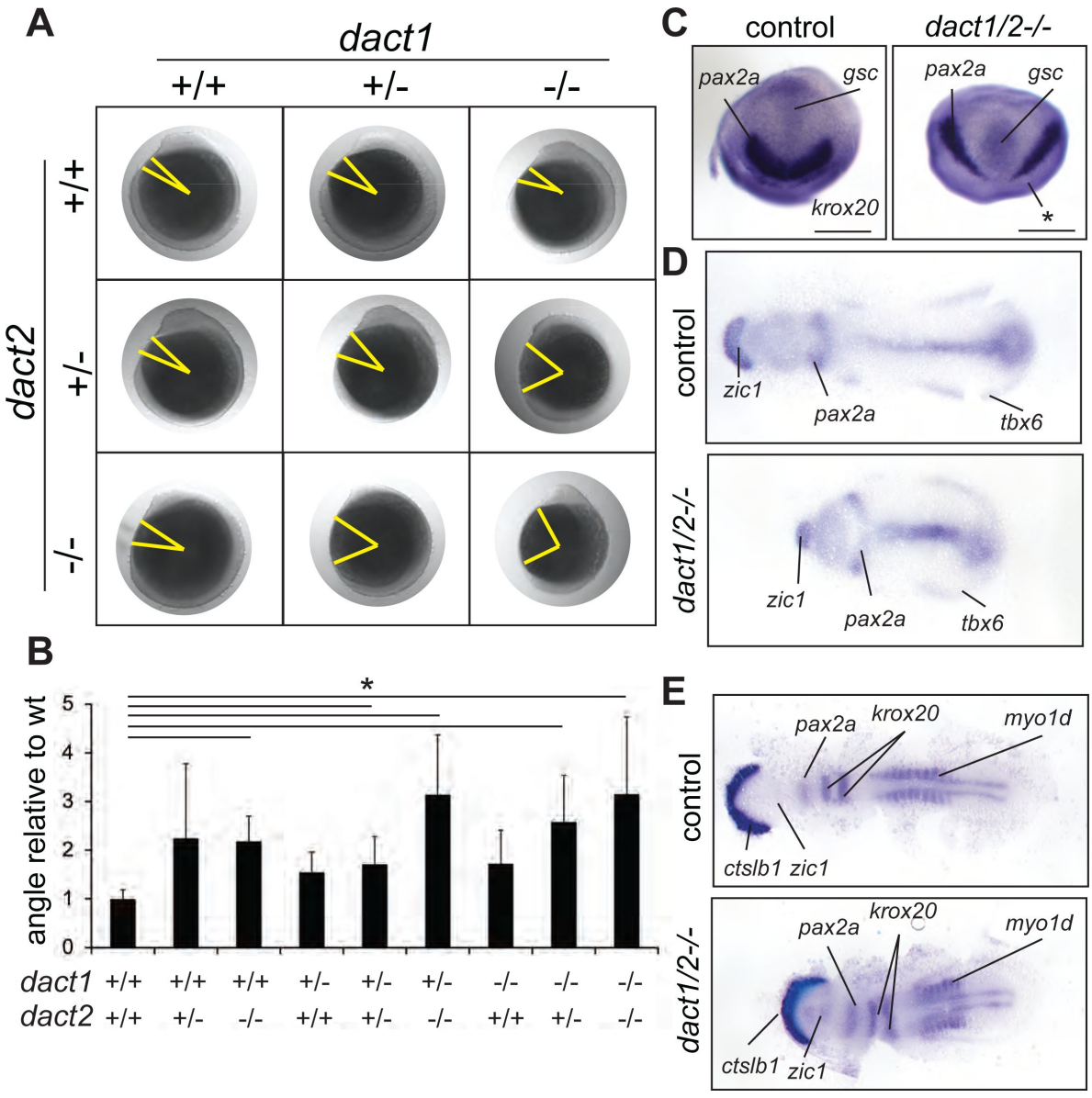


Figure 2.

Impaired convergent extension in *dact1* and *dact2* compound mutants. **A)** Inter-cross of compound heterozygotes yield embryos with different degrees of axis extension that correspond to the *dact1* and *dact2* genotypes. Representative lateral images of embryos at 12 hpf. The yellow line indicates body axis angle measured from the anterior point of the head, the center of the yolk, to the end of the tail. **B)** Quantification of body axis angle. Numbers represent the difference in angle relative to the average wildtype embryo. Asterisk indicates genotypes with angles significantly different from wildtype. ANOVA p&0.5 n= 3-21 embryos. **C)** Representative bud stage wildtype and *dact1-/-,dact2-/-* mutant embryos stained for *gsc* (prechordal plate), *pax2a* (midbrain/hindbrain boundary) and *krox20* (rhombomere 3). Asterisk indicates lack of *krox20* expression in *dact1-/-,dact2-/-* mutant. Scale bar = 200 µm **D)** Representative flat mounts of 1-2 ss wildtype and *dact1/2* mutant embryos stained for *zic1* (telencephalon), *pax2a* and *tbx6* (ventrolateral mesoderm). **E)** Representative flat mounts of 10 ss wildtype and *dact1/2-/-* mutant embryos stained for *ctsl1b* (hatching gland), *zic1*, *pax2a*, *krox20*, and *myo1d* (somites).

in *dact1-/-; dact2-/-* double mutants, which resulted in a shorter body axis. The aberrant CE and axis extension in the *dact1-/-;dact2-/-*phenotypes were similar to findings in other Wnt mutants, such as *slb* and *kyn* (Heisenberg, Tada et al. 2000, Topczewski, Sepich et al. 2001) in that the body axis is truncated upon segmentation.

dact1/*dact2* compound mutants exhibit axis shortening and craniofacial dysmorphology

Given the defective converge phenotype and shortened axis in the *dact* mutants during gastrulation, we examined the fish at 4 dpf for axis defects and for evidence of defective morphogenesis in the craniofacial cartilages. Craniofacial morphology is an excellent model for studying CE morphogenesis as many craniofacial cartilage elements develop through this cellular mechanism (Kamel, Hoyos et al. 2013, Mork and Crump 2015, Sisson, Dale et al. 2015, Rochard, Monica et al. 2016). No craniofacial phenotype was observed in *dact1* or *dact2* single mutants (data not shown). However, in-crossing to generate *dact1-/-; dact2-/-* compound homozygotes resulted in dramatic craniofacial malformation (**Fig. 3**). Specificity of this phenotype to *dact1/2* was confirmed via rescue with *dact1* or *dact2* mRNA injection (**Fig. S1**). The *dact1-/-;dact2-/-* mutant embryos exhibited fully penetrant midfacial hypoplasia (**Fig. 3A**) however the degree of eye field convergence in the midline varied between individuals. The forebrain protruded dorsally and the mouth opening and ventral cartilage structures were displaced ventrally (**Fig. 3A**). Alcian blue staining of cartilage elements revealed severe narrowing of the EP into a rod-like structure in 100 percent of double mutants, while the ventral cartilage elements were largely unaffected (**Fig. 3B**). Notably the trabeculae extending posteriorly from the EP and the rest of the posterior neurocranium exhibit wildtype morphology in *dact1/2*. This *dact1-/-;dact2-/-* double mutant phenotype is highly similar to that described for *wnt11f2* (*slb*) mutants, a key regulator of non-canonical Wnt signaling and CE (Kimmel, Miller et al. 2001).

As axis lengthening was found to be affected by loss of *dact1* and *dact2* (**Fig. 2A**) we measured body length in 5 dpf *dact1/2* compound mutants. Using the length of the vertebral spine as a measure of body length we found a trend (p=0.06) towards an effect of *dact1* and *dact2* on shortening of the body length. Similar to axis length during gastrulation/segmentation, the shortening was most pronounced in *dact1-/-; dact2-/-* double homozygous mutants versus wildtype clutch-mates (**Fig. 3C**, **Fig. S3A,B**).

As *wnt11f2* signals via disheveled and since dact proteins are known to interact with disheveled (Wong, Bourdelas et al. 2003, Zhang, Gao et al. 2006, Kivimae, Yang et al. 2011), it is suspected that dact has a role in wnt11f2 signaling. Combinatorial gene disruption with morpholinos showed that *dact2* morpholino exasperated the *wnt11* morpholino midfacial/eye fusion defect (Waxman, Hocking et al. 2004). We hypothesized that the shared phenotypes between *wnt11f2* and *dact1/2* mutants point to these genes acting in the same signaling pathway. To test for genetic epistasis between *wnt11f2*, *dact1*, and *dact2* genes we generated *wnt11f2-/-; dact1-/-; dact2-/-* triple homozygous mutants. If *wnt11f2* and *dact1/2* had independent developmental requirements, the *wnt11f2-/-; dact1-/-; dact2-/-* mutant may exhibit a phenotype distinct from *wnt11f2-/-* or *dact1-/-; dact2-/-* mutants. We found that the *wnt11f2-/-; dact1-/-; dact2-/-* triple homozygous mutant phenotype of the linear rod-like EP was the same as the *wnt11f2-/-* mutant or *dact1-/-;dact2-/-* double mutant, without exhibiting additional or neo-phenotypes in the craniofacial cartilages or body axis (**Fig. 3D,E**). This result supports *dact1 and dact2* acting downstream of *wnt11f2* signaling during ANC morphogenesis, where loss of *dact1/2* function recapitulates a loss of *wnt11f2* signaling.

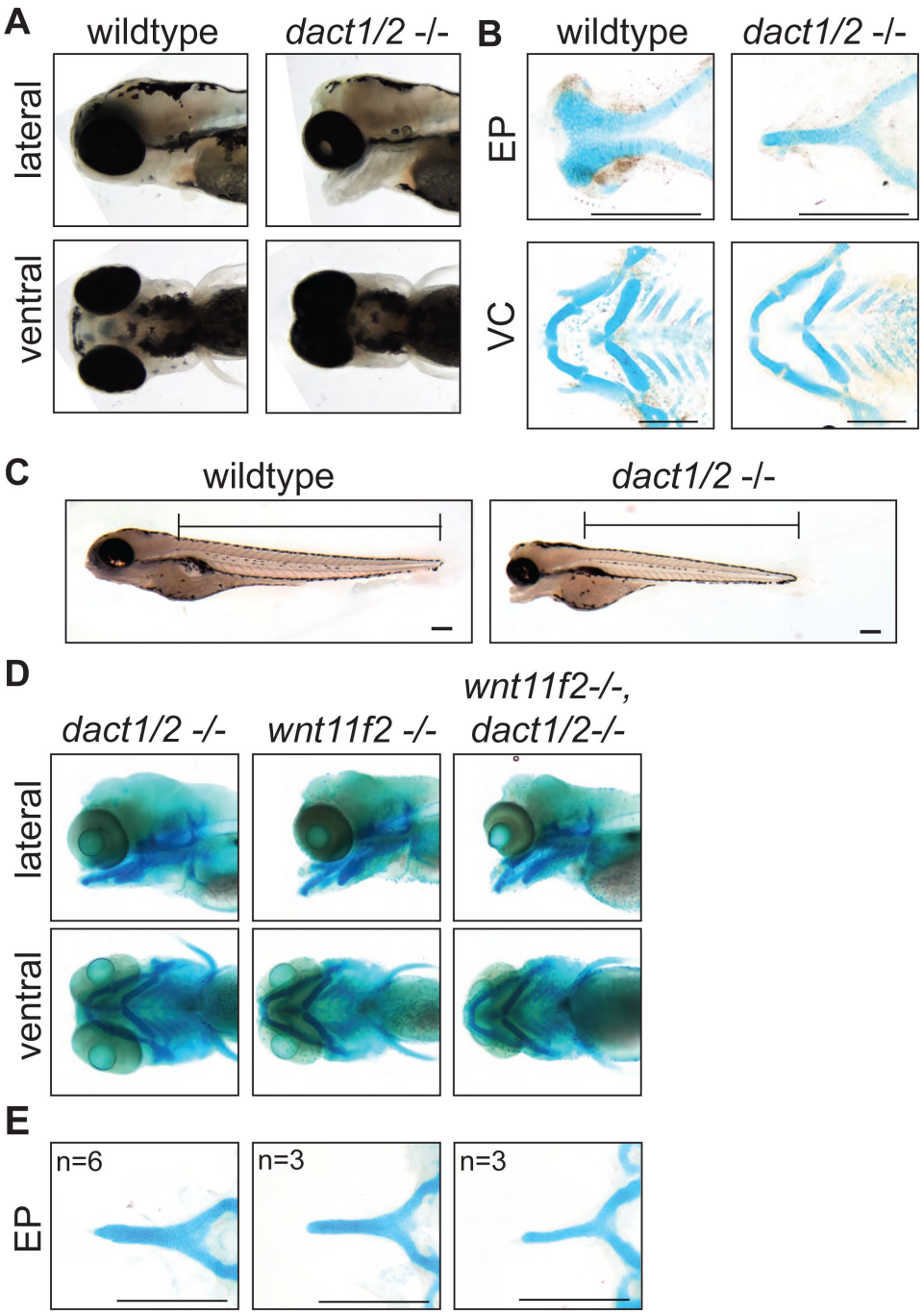


Figure 3.

Midface development requires *dact1* and *dact2*. **A)** Representative brightfield images of wildtype and *dact1/2-/-* compound mutants at 4 dpf. 100 individuals were analyzed from a *dact1*+/-,*dact2*+/-double het cross. Lateral and ventral views show d*act1/2-/-* compound mutants have a hypoplastic midface, medially displaced eyes, and a displaced lower jaw. **B)** Representative flat-mount images of Alcian blue stained ethmoid plate (EP) and visceral cartilage (VC) elements from 4 dpf wildtype and d*act1/2-/-* compound mutants. d*act1/2-/-* mutants have a rod-shaped EP with no distinct lateral and medial elements. No obvious differences were found in *dact1/2* mutant VC. **C)** Representative brightfield image of 4dpf wildtype and *dact1/2-/-* mutant. Bar indicates vertebral spine length. Scale bar: 100 µm. **D)** Representative images of wildtype and dact1/2-/-mu Alcian blue stained *dact1/2-/-*, *wnt11f2-/-*, and *wnt11f2-/-*,*dact1/2-/-*compound mutants. Embryos resulted from a *dact1+/-*,*dact2+/-*,*wnt11f2+/-* in-cross. Lateral and ventral views show similar craniofacial phenotypes in each mutant. **E)** Representative flat-mount images of Alcian blue stained EP show a similar phenotype between *dact1/2-/-*, *wntllf2-/-*, and *wnt11f2-/-*,*dact1/2-/-*compound mutants. Scale bar: 200 µm.

Lineage tracing of *dact1/2* mutant NCC movements reveals their ANC composition

The EP forms from the convergence of a central frontal prominence-derived structure with bilateral maxillary prominence-derived elements (Wada, Javidan et al. 2005, Swartz, Sheehan-Rooney et al. 2011, Dougherty, Kamel et al. 2012, Mork and Crump 2015, Rochard, Monica et al. 2016).The stereotypic convergent migration of cranial neural crest cells and their derivatives presents an excellent model to examine CE movements and their effects on tissue morphogenesis. The zebrafish EP is formed from the joining of a midline frontal prominence derived from the anteromost cranial neural crest (NCC) cell population that migrate over the eyes and turn caudally, to join paired lateral maxillary prominences derived from the second stream of cranial NCC population that migrate rostrally (Kimmel, Miller et al. 2001, Wada, Javidan et al. 2005, Schilling and Le Pabic 2009, Dougherty, Kamel et al. 2012, Mork and Crump 2015). The EP that forms is a planar fan-shaped structure where we and others have shown that the morphology is governed by Wnt signaling (Kimmel, Miller et al. 2001, Rochard, Monica et al. 2016).

Given the rod-like EP we observed in the *dact1-/-; dact2-/-* double mutants, we hypothesized that the dysmorphology could be due to aberrant migration of the anteromost midline stream of cranial NCCs resulting in fusion of the lateral maxillary components. Conversely, an abrogated contribution from the second paired stream of maxillary NCCs could lead to an EP composed entirely of the medial component. To distinguish between these possibilities, we carried out lineage tracing of the cranial NCC populations in wildtype and *dact1/2* mutants. The *dact1/2* compound mutants were bred onto a *sox10:kaede* transgenic background, where we and others have shown that the *sox10* reporter is a reliable driver of cranial neural crest labeling (Wada, Javidan et al. 2005, Dutton, Antonellis et al. 2008, Swartz, Sheehan-Rooney et al. 2011, Dougherty, Kamel et al. 2012, Kague, Gallagher et al. 2012, Mork and Crump 2015). Cranial NCC populations in wildtype and *dact1/2* mutants were targeted at 19 hpf to photoconvert Kaede reporter protein in either the anterior cranial NCCs that contribute to the frontal prominence, or the second stream of NCCs that contribute to the maxillary prominence, where the labeled cells were followed longitudinally over 4.5 days of development (**Fig. 4**). We found that the anterior NCCs of wildtype embryos migrated antero-dorsally to the eye and populated the medial EP. To our surprise, the anterior cranial NCC also migrated to contribute to the median element of the rod-like EP, suggesting the complex anterior then caudal migration of the anterior NCC is not disrupted by *dact1/2* mutation (**Fig. 4A**, arrows). This finding is in contrast to lineage tracing in another midline mutant with a similarly shaped rod-like EP, the *syu* (*sonic hedgehog* null) mutant, where the anterior NCCs failed to populate the ANC (Wada, Javidan et al. 2005).

Next, the second stream of NCC population that contribute to the maxillary prominence was labeled, where they migrate and contribute to the lateral element of the EP as expected in the wildtype (**Fig 4B**). When the second stream of cranial NCC were labeled and followed in the *dact1/2* mutants, the cells were found to migrate normally up to 36 hpf, but did not ultimately populate the EP in the mutant (arrows), suggesting that the morphological movements after NCC migration were disrupted by loss of *dact1/2* function. These results suggest that NCC migration itself is not regulated by *dact1/2* but that loss of *dact1/2* hinders the second stream of NCCs' ability to populate the ANC. These results show that the rod-like EP can be formed from 2 different NCC origins, where in the *dact1/2* mutants the EP is contributed by the anteromost frontonasal NCCs, in contrast to the similar rod-shaped EP of the *syu* mutants that is formed from the more posterior stream of maxillary NCCs (Wada, Javidan et al. 2005).

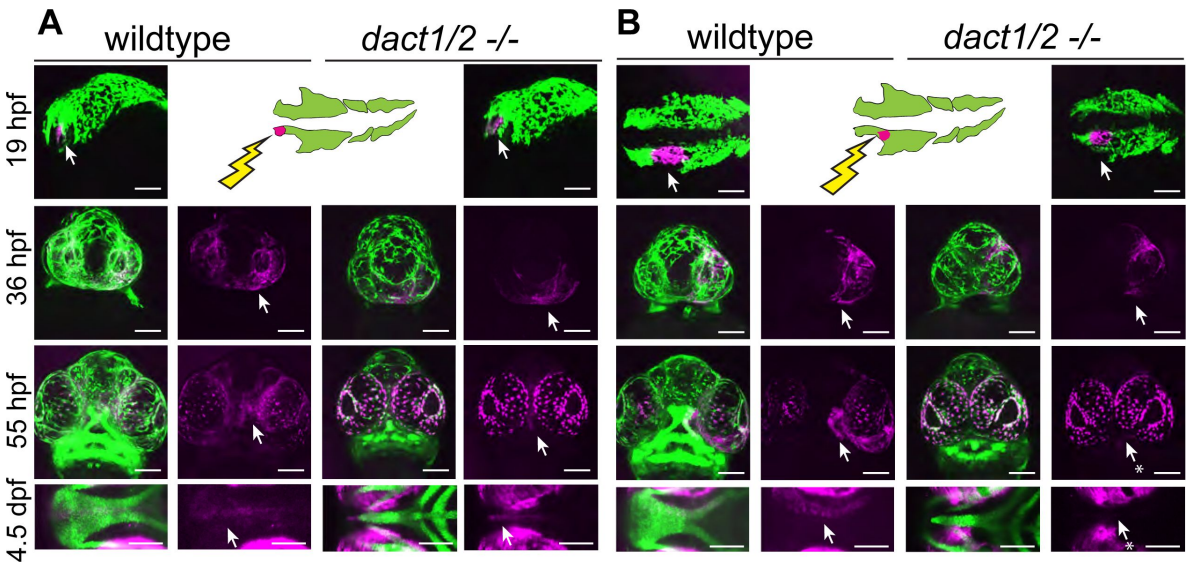


Figure 4.

Anterior neural crest cells of the *dact1/2-/-* mutant migrate to the midline and populate the dysmorphic ethmoid plate. Lineage tracing of wildtype and *dact1/2-/-* double mutant zebrafish embryos using Tg(*sox10*:kaede) line. *sox10*:kaede fluorescence is shown in green and photo-converted kaede is shown in magenta and highlighted with an arrow. Asterisks indicate that the cell population is absent. **A,B)**19 hpf embryo sagittal views showing photoconversion of anterior-most neural crest population. At 36 hpf frontal images show the migration of photoconverted neural crest cells to the frontal prominence in wildtype and *dact1/2-/-* double mutants. At 55 hpf, frontal images show photoconverted neural crest cells populating the region of the developing ANC in wildtype and *dact1/2-/-* mutants. At 4.5 dpf ventral images show photoconverted neural crest cells populating the medial ethmoid plate in wildtype. Similarly, neural crest cells in *dact1/dact2-/-* mutants populate the rod-shaped ethmoid plate. Scale bar:100 μm. Representative images of 3 individual experiments.

Genetic interaction of *dact1/2* with *gpc4* and *wls* to determine facial morphology

Given the role of Dact/dapper as modifiers of Wnt signaling, we hypothesized that genetic interaction of *dact1/2* with *wls* and *gpc4* will modify facial morphology. Gpc4 is a glycoprotein that binds Wnt ligands and modulates Wnt signaling. *gpc4* zebrafish mutants (*kny*) have impaired CE which leads to a shortened body axis (Topczewski, Sepich et al. 2001). Wls is a posttranslational modifier of Wnt ligands which promotes their secretion (Banziger, Soldini et al. 2006, Bartscherer, Pelte et al. 2006). We previously described that these components of the Wnt/PCP pathway (*gpc4* receptor, *wls* intracellular ligand chaperon, and Wnt ligands *wnt9a* and *wnt5b)* are required for craniofacial morphogenesis, where each gene affects particular morphologic aspects of chondrocytes arrangement in the cardinal axis of the ANC and Meckel's cartilage (Rochard, Monica et al. 2016, Ling, Rochard et al. 2017). Using the EP as a morphologic readout, we examined the genetic interaction of *dact1* and *dact2* with *wls* and *gpc4*. Compound mutants of *dact1*, *dact2*, *gpc4* or *wls* were generated by breeding the single alleles. Compared to wildtype ANC morphology, abrogation of *gpc4* led to increased width in the transverse axis, but shorter in the antero-posterior axis (Rochard, Monica et al. 2016). Disruption of *wls* leads to ANC morphology that is also wider in the transverse dimension, but to a lesser degree than observed in *gpc4*. Additionally, in the *wls* mutant, chondrocytes stack in greater layers in the sagittal axis (Rochard, Monica et al. 2016).

Disruption of *gpc4* or *wls* in addition to *dact1/2* generated EP morphology that contained phenotypic attributes from each single mutant, so that the resultant ANC morphology represented a novel ANC form. The EP of a triple homozygous *dact1-/-; dact2-/-; gpc4-/-* mutant was triangular, wider in the transverse axis and shorter in the antero-posterior axis compared to the rod-like ANC observed in the *dact1-/-;dact2-/-* double mutant (**Fig. 5A,B**). Similarly, the ANC of a triple homozygous *dact1-/-; dact2-/-; wls-/-* mutant was in the shape of a rod, shorter in the antero-posterior axis and thicker in the sagittal axis compared to the *dact1-/-;dact2-/-* double mutant, reflecting attributes of the *wls* mutant (**Fig. 5C,D**). In addition to the EP phenotypes, the triple homozygous *dact1-/-; dact2-/-; gpc4-/-* mutant also had a short body axis and truncated tail similar but more severe than the *gpc4* mutant (**Fig. 5A**). Since compound disruption of *dact1*, *dact2*, and *gpc4* or *wls* resulted in a new phenotype we conclude that these genes function in different components of Wnt signaling during craniofacial development.

As we analyzed the subsequent genotypes of our *dact1/dact2/gpc4* triple heterozygote in-cross we gleaned more functional information about *dact1* and *dact2*. We found that *dact1* heterozygosity in the context of *dact2-/-; gpc4-/-* was sufficient to replicate the triple *dact1/dact2/gpc4* homozygous phenotype (**Fig. 5E**). In contrast, *dact2* heterozygosity in the context of *dact1-/-; gpc4-/-* double mutant produced ANC in the opposite phenotypic spectrum of ANC morphology, appearing similar to the *gpc4-/-* mutant phenotype (**Fig. 5E**). These results show that *dact1* and *dact2* do not have redundant function during craniofacial morphogenesis, and that *dact2* function is more indispensable than *dact1*. These results also suggest that *dact1* and *gpc4* may have overlapping roles in craniofacial development.

dact1/2 and *gpc4* regulate axis extension via overlapping and distinct cellular pathways

Our analyses of axis extension and the hallmarks of a CE defect (namely decreased length and increased width between early tissues) demonstrate that *dact1* and/or *dact2* are required for CE and anterior-posterior axis lengthening during gastrulation (**Fig. 3**). An axis lengthening and CE defect has also been described in *gpc4* (aka kny) mutants (Topczewski, Sepich et al. 2001). We also observe a defect in axis lengthening in *gpc4-/-* in our hands (representative image **Fig. 6A**) that is grossly similar to the *dact1/2-/-* mutants. Interestingly, the midfacial hypoplasia of the

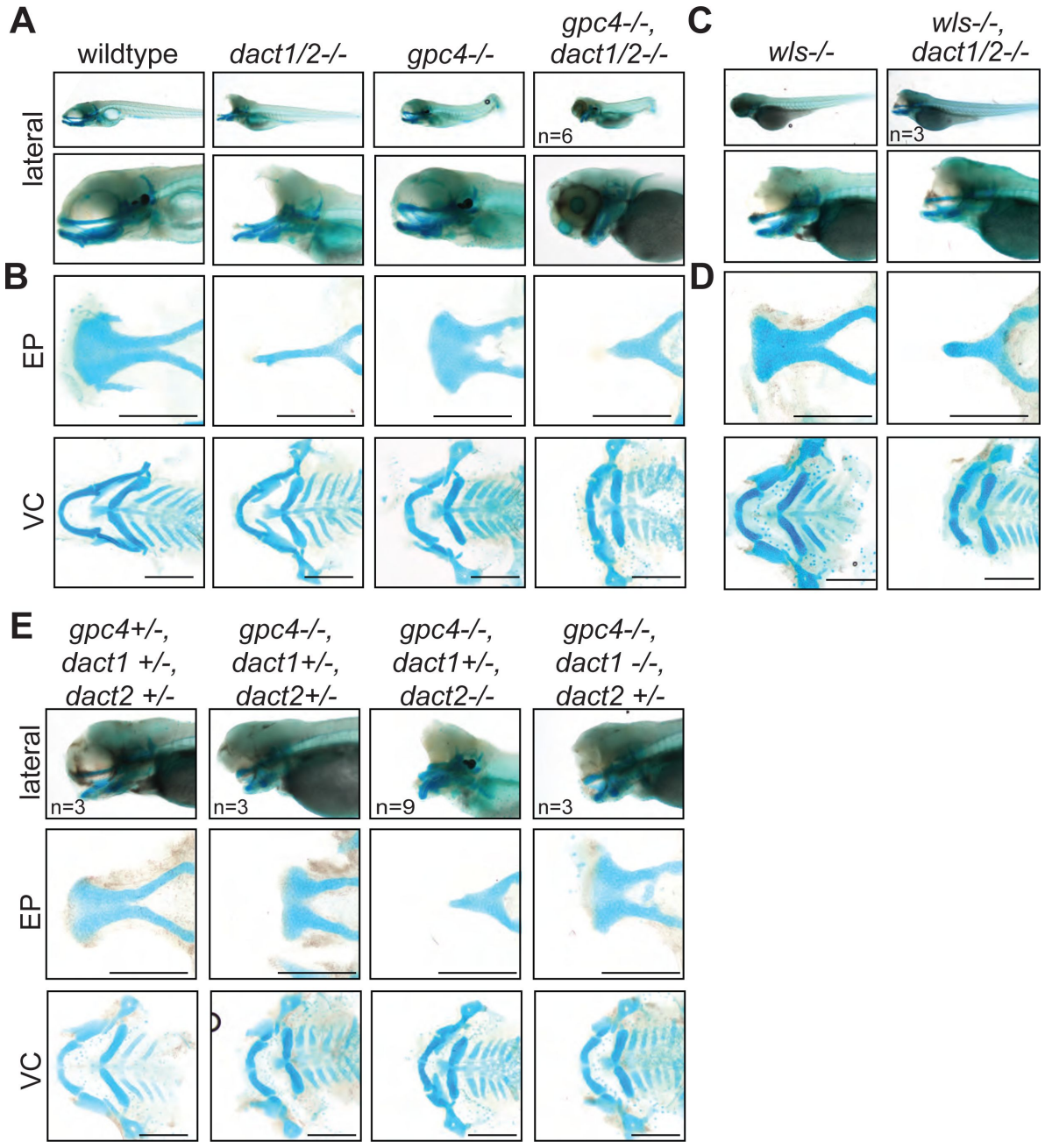


Figure 5.

A nonoverlapping functional role for *dact1*, *dact2*, and *gpc4* and *wls*. **A)** Representative Alcian blue stained wholemount images of wildtype, *dact1/2-/-* double mutant, *gpc4-/-* mutant, and *gpc4-/-,dact1/2-/-* triple mutants at 4 dpf. Low magnification lateral images of embryos showing tail truncation in *dact1/2-/-* mutants, shortened and kinked tail in *gpc4-/-* mutants, and a combinatorial effect in *gpc4-/-,dact1/2-/-* triple mutants. Higher magnification lateral images show a shortened midface and displaced lower jaw in *dact1/2-/-* mutants, a shortened midface in *gpc4-/-* mutant, and a combinatorial effect in *gpc4-/-,dact1/2-/-* triple mutants. **B)** Representative flat-mount images of dissected Alcian blue-stained cartilage elements. *dact1/2-/-* mutants have a narrow rod-shaped ethmoid plate (EP) while *gpc4-/-* mutants have a broad and shortened EP. *dact1/2/gpc4* triple mutants have a combinatorial effect with a short, broad rod-shaped EP. In ventral cartilages (VC), *dact1/2-/-* mutants have a relatively normal morphology while Meckel's cartilage in *gpc4-/-* mutants and *gpc4-/-,dact1/2-/-* triple mutants is truncated. **C,D)** Same as above except *wls-/-* mutant and *wls-/-,dact1/2-/-* triple mutant, with similar findings. **E)** Combinatorial genotypes of *dact1*, *dact2*, and *gpc4*. *dact2*-/-contributed the *dact/gpc4* compound phenotype while *dact1-/-* did not. Scale bar: 200 μm.

wnt11f2 (slb) mutant has been attributed to a defect in axis extension and anterior neural plate patterning (Heisenberg and Nusslein-Volhard 1997), whereas defective axis extension does not lead to midfacial hypoplasia in the *gpc4-/-* mutant. Therefore, we hypothesized that by comparing and contrasting the gene expression changes in *dact1/2* versus *gpc4* mutants during axis extension we could identify cell programs specifically responsible for the anterior axis defect and subsequent midfacial hypoplasia. We performed single-cell transcriptional analysis to compare *dact1/2* mutants, *gpc4* mutants, and wildtype embryos during the segmentation stage. Single-cell encapsulation and barcoded cDNA libraries were prepared from individual dissociated 4 ss wildtype, *dact1-/-;dact2-/-* compound mutant and *gpc4-/-* mutant embryos using the 10X Genomics Chromium platform and Illumina next-generation sequencing. Genotyping of the embryos was not possible but quality control analysis by considering the top 2000 most variable genes across the dataset showed good clustering by genotype, indicating the reproducibility of individuals in each group. Twenty clusters were identified using Louvain clustering and identity was assigned by reviewing cluster-specific markers in light of published expression data (Farrell, Wang et al. 2018, Farnsworth, Saunders et al. 2020, Bradford, Van Slyke et al. 2022) (**Fig. 6B,C**). Qualitatively, we did not observe any significant difference in cluster abundance between genotype groups (**Fig. S4**). We found that *dact1*, *dact2*, and *gpc4* were detected at various levels across clusters, though *dact1* expression was lower than *dact2* (**Fig. 6D**), consistent with what we observed in RNA wholemount ISH analysis (**Fig. 1**).

To assess the relative differences in gene expression between genotype groups, we merged clusters into broader cell lineages: ectoderm, axial mesoderm, and paraxial mesoderm (**Fig. 7A**). We focused on these cell types because they contribute significantly to CE processes and axis establishment. For each of these cell lineages, we performed independent pseudo-bulk differential expression analyses (DEA) of wildtype vs. *dact1-/-;dact2-/-* mutant and wildtype vs. *gpc4-/-* mutant (**Fig. 7A**). In all 3 cases, we found differentially expressed genes (DEGs) that were commonly in *dact1-/-;dact2-/-* and *gpc4-/-* mutant relative to wildtype (**Fig. 7B**). To address the hypothesis that *dact1* and *dact2* regulate molecular pathways distinct from those regulated by *gpc4* we also identified genes that were differentially expressed only in *dact1/2-/-* mutants or only in *gpc4-/-* mutants (**Fig. 7B**). Functional analysis of these DEGs found unique enrichment of intermediate filament genes in *gpc4-/-* whereas *dact1/2-/-* mutants had enrichment for pathways associated with proteolysis (**Fig. 7C**, **S5**). Enrichment for pathways associated with calcium-binding were found in both *gpc4-/-* and *dact1/2-/-*, although the specific DEGs were distinct (**Fig. S5**). We performed functional analyses specifically for genes that were differentially expressed in *dact1-/-;dact2-/-* mutants, but not in *gpc4-/-* mutants, and found enrichment in pathways associated with proteolysis (**Fig. 7B**) suggesting a novel role for Dact in embryogenesis.

Interrogation of *dact1-/-;dact2-/-* mutant-specific DEGs found that the calcium-dependent cysteine protease *calpain 8* (*capn8*) was significantly overexpressed in *dact1-/-;dact2-/-* mutants in paraxial mesoderm (103 fold), axial mesoderm (33 fold), and in ectoderm (3 fold; **Fig. 7A**). We also found that loss of *dact1/2* causes significant changes to *capn8* expression pattern (**Fig. 8A**). Whereas *capn8* gene expression is principally restricted to the epidermis of wildtype embryos, loss of *dact1/2* leads to significant expansion of ectopic *capn8* gene expression in broader cell types such as in mesodermal tissues (**Fig. 8A**). We corroborated this finding with wholemount RNA ISH for *capn8* expression in wildtype versus *dact1/2-/-*12 hpf embryos (**Fig. 8B**). Expression of *smad1* was also found to be decreased uniquely in the ectoderm of *dact1/2-/-*embryos relative to wildtype (**Fig. 7A**), however this finding was not investigated further,

Capn8 is considered a “classical” calpain, with domain homology similar to *Capn1* and *Capn2* (Macqueen and Wilcox 2014). In adult human and mouse tissue, *Capn8* expression is largely restricted to the gastrointestinal tract (Sorimachi, Ishiura et al. 1993, Macqueen and Wilcox 2014), however embryonic expression in mammals has not been characterized. Proteolytic targets of Capn8 have not been identified, however, other classical calpains have been implicated in Wnt and cell-cell/ECM signaling (Konze, van Diepen et al. 2014), including in Wnt/Ca^{+2}

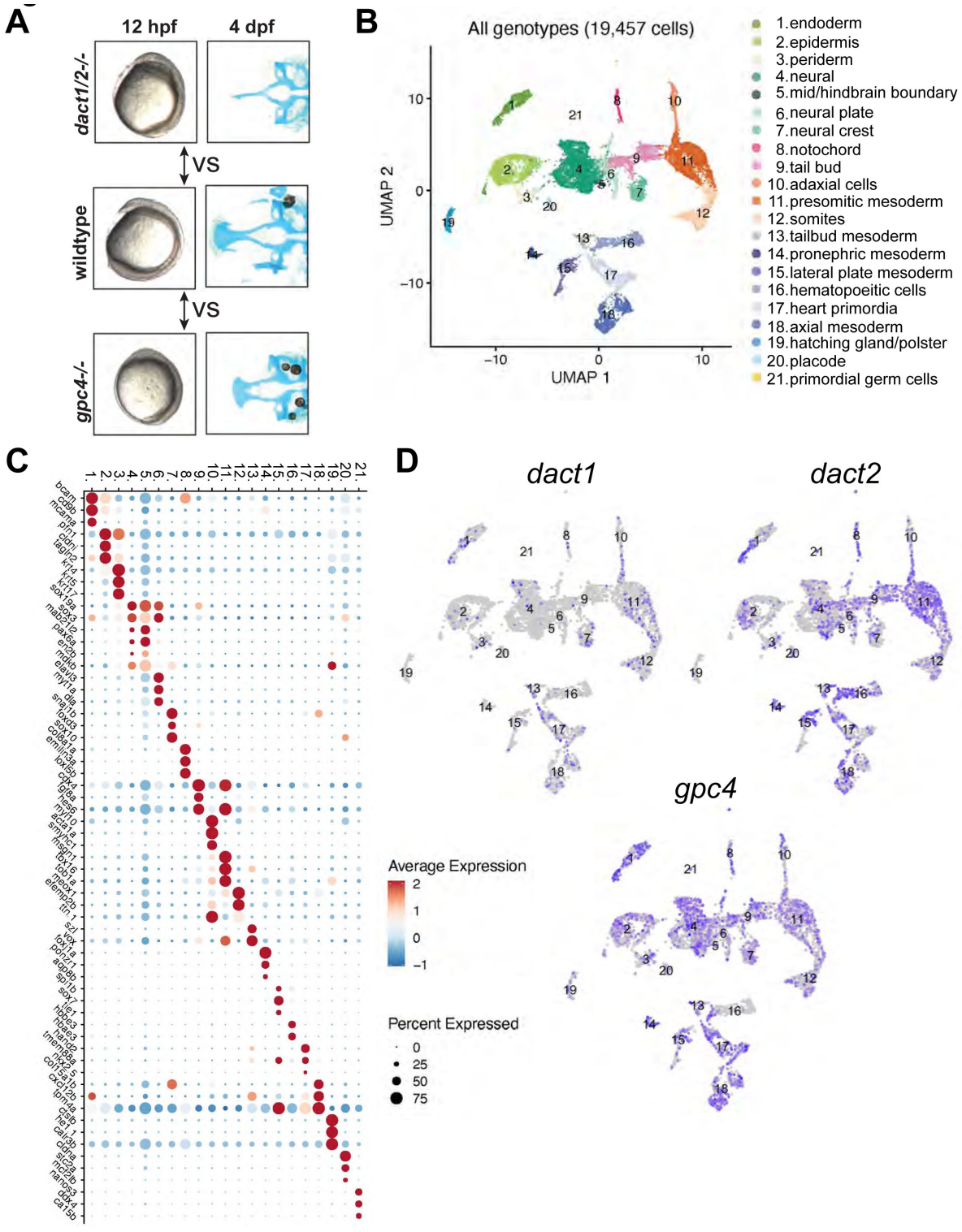


Figure 6.

Single-cell RNAseq of 4 ss wildtype, *dact1/2-/-* mutant and *gpc4-/-* mutants. **A)** Summary schematic showing similar phenotypes in *dact1/2-/-* and *gpc4-/-* mutants at 12 hpf and divergent phenotypes at 4 dpf. Single-cell RNAseq was performed during axis extension to compare and contrast *dact1/2-/-* and *gpc4-/-* transcriptional programs. UMAP showing cluster identification. **B)** UMAP of cell clusters identified by Single-cell RNAseq. **C)** Dot plot showing the most differentially expressed genes between clusters. **D)** UMAP showing *dact1*, *dact2*, and *gpc4* expression in wildtype embryos.

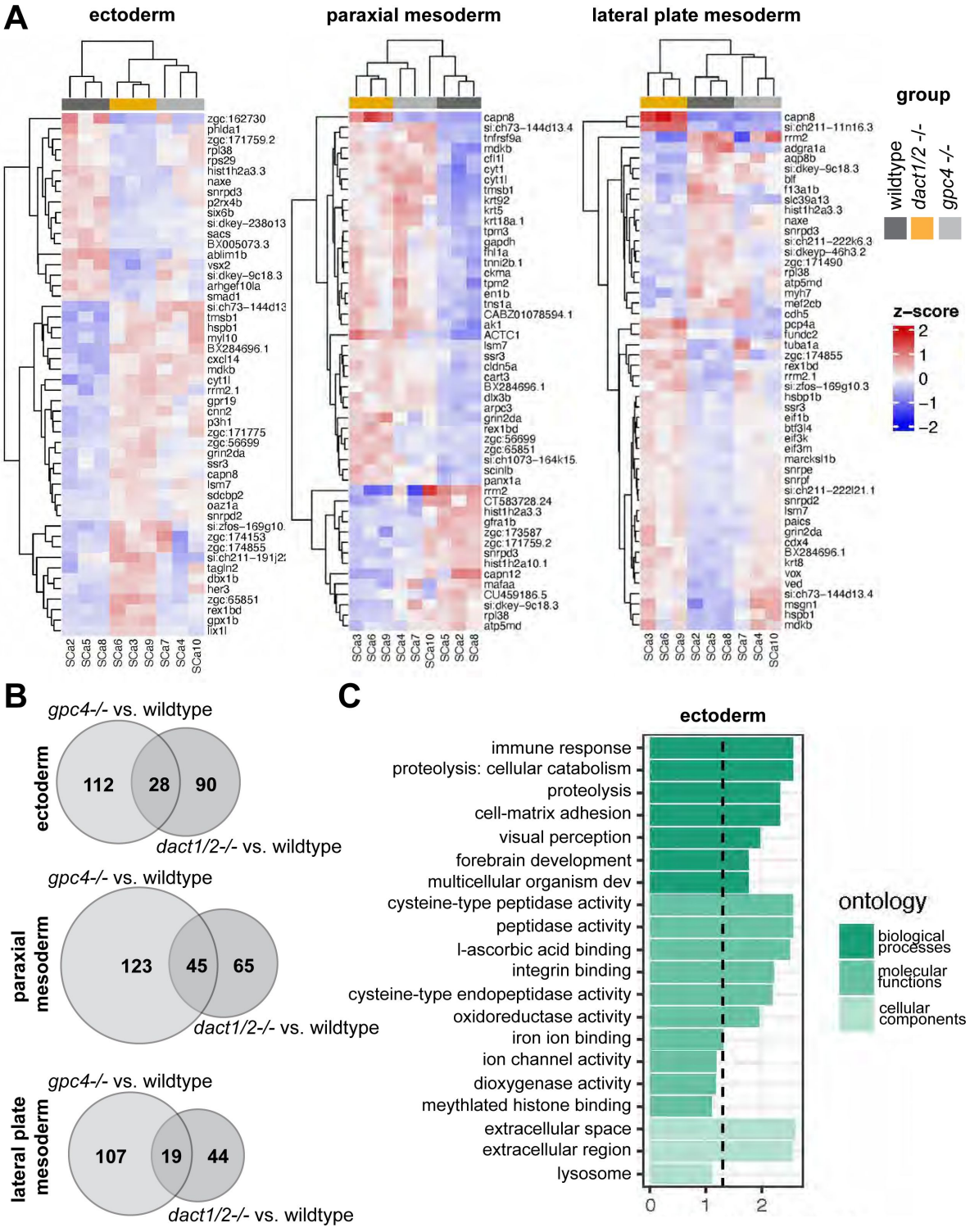


Figure 7.

Pseudobulk differential expression analysis of single-cell RNAseq data. **A)** Heatmaps showing the 50 most differentially expressed genes in 3 major cell types; ectoderm (clusters 4,5,6,7), paraxial mesoderm (clusters 10,11,12), and lateral plate mesoderm (clusters 15, 16, 17,18) between *dact1/2-/-* mutants and wildtype and *gpc4-/-* mutants and wildtype. **B)** Venn diagrams showing unique and overlapping DEGs in *dact1/2-/-* and *gpc4-/-* mutants. **C)** GO analysis of *dact1/2-/-* mutant-specific DEGs in lateral plate mesoderm showing enrichment for proteolytic processes.

regulation of CE in Xenopus (Zanardelli, Christodoulou et al. 2013). To determine whether the *dact1-/-;dact2-/-* mutant craniofacial phenotype could be attributed to *capn8* overexpression, we performed injection of *capn8* or *gfp* control mRNA into 1 cell-stage zebrafish embryos. In wildtype zebrafish, exogenous *capn8* mRNA caused the distinct *dact1/2*-/-craniofacial phenotype including a rod-like ANC at a very low frequency (1 in 142 injected embryos). This phenotype was never observed in wildtype larvae, or when wildtype embryos were injected with an equal concentration of *gfp* mRNA (0 in 192 injected embryos) (**Fig. 8C**). When mRNA was injected into embryos generated from *dact1/2*+/-interbreeding, *capn8* caused a significant increase in the number of larvae with the mutant craniofacial phenotype (**Fig. 8D**, 0.0% vs. 7.5%). These findings suggest a new contribution of capn8 to embryonic development as well as anterior neural plate patterning and craniofacial development. Further, the regulation of *capn8* by *dact* may be required for normal embryogenesis and craniofacial morphogenesis.

Discussion

In this study, we examined the genetic requirement of *dact1* and *dact2* during early embryogenesis and craniofacial morphogenesis in zebrafish. Wnt signaling is central to the orchestration of embryogenesis and numerous proteins have been identified as modulators of Wnt signaling, including *Dact1* and *Dact2* (Cheyette, Waxman et al. 2002). Several studies across *Xenopus*, zebrafish, and mouse have ascribed roles to *dact1* and *dact2*, including both promoting and antagonizing Wnt signaling, depending on the developmental context (Cheyette, Waxman et al. 2002, Gloy, Hikasa et al. 2002, Waxman, Hocking et al. 2004, Gao, Wen et al. 2008, Wen, Chiang et al. 2010, Ma, Liu et al. 2015, Lee, Cheong et al. 2018). Here, we show that *dact1* and *dact2* are required for axis extension during gastrulation and show an example of CE defects during gastrulation associated with craniofacial defects. During axis extension, we show that genetic disruption of *dact2*, but not *dact1*, resulted in a significantly shortened axis relative to wildtype. This result is similar to what was previously found using morpholinos to disrupt *dact1* and *dact2* (Waxman, Hocking et al. 2004). Interestingly, genetically disrupted mutants of *dact1* or *dact2* developed to be phenotypically normal whereas *dact1/2* compound mutants displayed a severe dysmorphic craniofacial phenotype. Again, this is largely similar to the previous morpholino study that found disruption of each gene to cause only a slight and occasional dysmorphic cranial phenotype at 24 hpf (Waxman, Hocking et al. 2004). Notably, embryos injected with a mixture of *dact1* and *dact2* morpholino were not characterized after 10 ss, and the singly injected embryos were not characterized after 24 hpf (Waxman, Hocking et al. 2004). Therefore, by analyzing genetic mutants of dact1 and dact2 our findings have largely validated the previous morpholino literature as well as added new data on later developmental outcomes.

The gene expression and genetic epistasis experiments carried out here support that the *dact* paralogs are not redundant and have unique functions during different stages of embryonic and larval development. We observed that *dact1* and *dact2* have distinct spatiotemporal expression patterns throughout embryogenesis, suggesting unique roles for each paralog in developmental processes. Differential expression of *Dact1* and *Dact2* was also described during odontogenesis in mice (Kettunen, Kivimae et al. 2010). This aligns with previous findings of differential roles of *dact1* and *dact2* in canonical vs. non-canonical Wnt signaling (Waxman, Hocking et al. 2004) and a specific role for *dact2*, but not *dact1*, in TGF-beta signaling (Su, Zhang et al. 2007, Schubert, Sobreira et al. 2014). However, the lack of a resultant phenotype upon genetic ablation of *dact1* or *dact2* individually suggests the capability of functional compensation. This is puzzling given their distinct expression patterns and needs to be examined further.

We found that *dact1* and *dact2* contribute to axis extension, and their compound mutants exhibit a shortened and widened body axis that is consistent with a CE defect during gastrulation. This finding aligns with previous studies that have implicated *dact1* and *dact2* in non-canonical Wnt signaling and regulation of embryonic axis extension (Waxman, Hocking et al. 2004). Based on

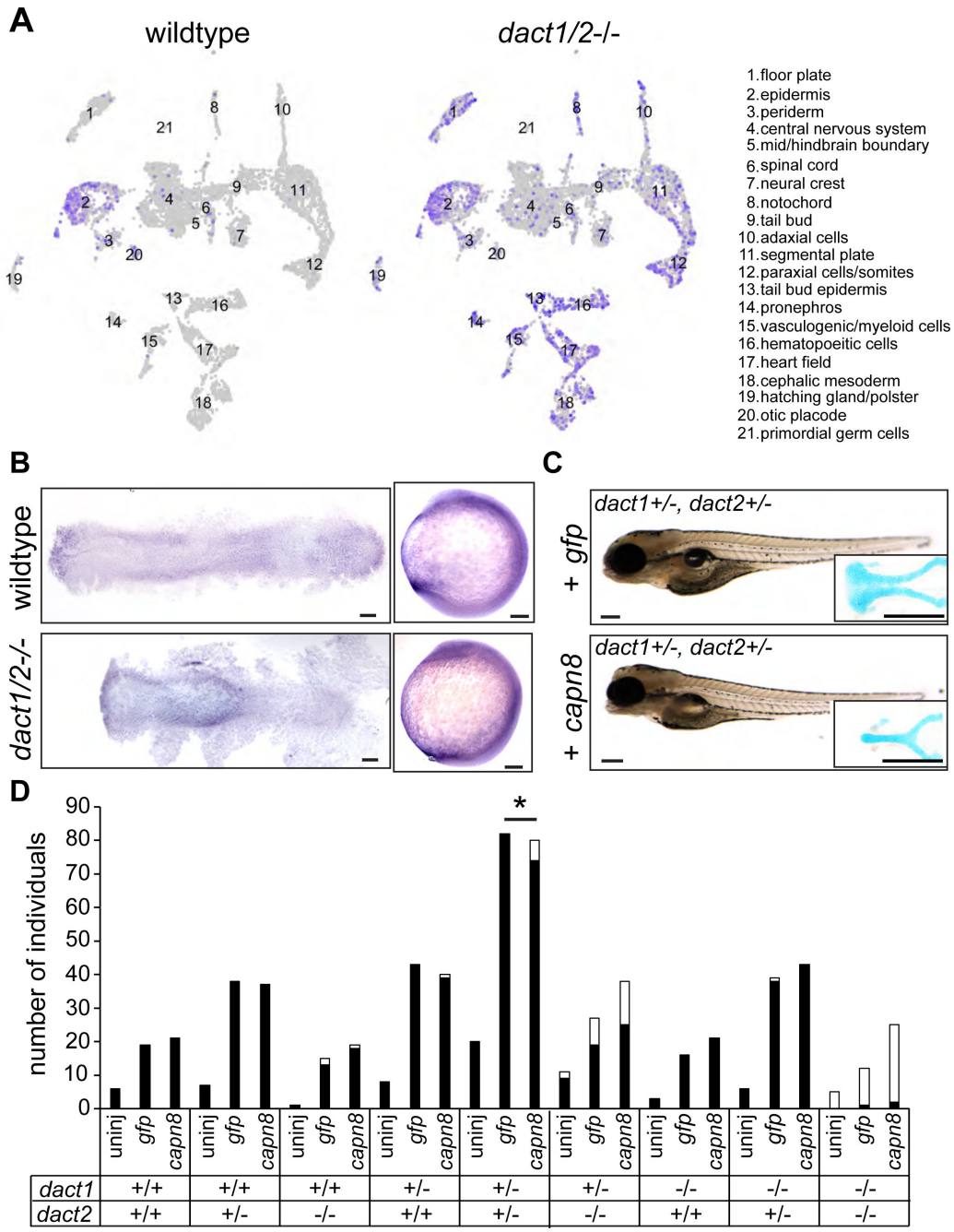


Figure 8.

Expression of *capn8* is significantly dysregulated in *dact1/2-/-* mutants. **A)** Single-cell RNAseq gene expression analysis of *capn8* in wildtype and *dact1/2-/-* mutants. In wildtype embryos, *capn8* expression is restricted predominantly to the epidermis whereas *capn8* is widely expressed throughout the embryo in *dact1/2-/-* mutants, especially in the mesoderm. **B)** Wholemount *in situ* hybridization of *capn8* expression in wildtype and *dact1/2-/-* mutant embryos at 2 ss. Staining corroborates the single-cell RNAseq data, with expanded ectopic expression of *capn8* throughout the embryo. Flat mounts are oriented anterior to the left. Scale bar: 100 µm. **C)** Brightfield images and alcian blue staining of the ethmoid plate show ectopic expression of *capn8* mRNA (200pg) at the 1-cell stage in *dact1+/-,dact2+/-* embryos recapitulates the *dact1/2-/-* compound mutant craniofacial phenotype. The mutant craniofacial phenotype did not manifest in gfp mRNA (200 pg) injected 1-cell stage *dact1+/-,dact2+/-* embryos. Scale bar: 100 µm **D)** Quantification of mutant and normal craniofacial phenotype in 4 dpf larvae after mRNA injection at the 1-cell stage. Larvae were derived from *dact1+/-,dact2+/-* interbreeding. Larvae were uninjected or injected with 200 pg *gfp* control or *capn8* mRNA. A Fisher exact test showed a significant effect of *capn8* mRNA injecting in the *dact1/2* double heterozygotes. p= 0.013.

our gene expression and combinatorial genetic analyses, we offer the hypothesis that *dact1* expression in the paraxial mesoderm is required for dorsal CE during gastrulation through its role in noncanonical Wnt/PCP signaling, similar to the defect observed upon *gpc4* disruption. Conversely, we posit that *dact2* functions in the prechordal mesoderm to promote anterior migration during gastrulation, a function which has also been ascribed to *wntllf2* (Heisenberg and Nusslein-Volhard 1997). It is only upon loss of both *dact1* and *dact2* functions that the axis is significantly truncated and a craniofacial malformation results. Further experiments with spatially restricted gene ablation or cell transplantation are required to test this hypothesis.

Our results underscore the crucial roles of *dact1* and *dact2* in embryonic development and suggest a connection between gastrulation movements and subsequent craniofacial morphogenesis. Our finding that in *dact1-/-;dact2-/-* compound mutants the first stream of CNCC migrate and contribute to the ANC, while the second stream fails to contribute suggests the possibility of an anatomical barrier to migration, rather than an autonomous defect of the CNCCs. Disruption of the sonic hedgehog signaling pathway in zebrafish results in a similar phenotype to *dact1-/-;dact2-/-* and *wntllf2-/-* mutants where the eyes converge medially and the EP narrows to a rod shape. Interestingly, lineage tracing analysis in hedgehog-disrupted embryos found the rod-like EP to consist solely of second stream-derived CNCCs (Wada, Javidan et al. 2005). This is in contrast to the *dact1-/-;dact2-/-* mutants, demonstrating two different cellular mechanisms that result in a similar anatomical dysmorphology. It will be important to test the generality of this phenomenon and determine if other mutants with craniofacial abnormalities have early patterning differences. Further, a temporally conditional genetic knockout is needed to definitively test the connection between early and later development.

By comparing the transcriptome across different Wnt genetic contexts, i.e. *gpc4-/-*with that of the *dact1-/-;dact2-/-* compound mutant, we identified a novel role for *dact1/2* in the regulation of proteolysis, with significant misexpression of *capn8* in the mesoderm of *dact1-/-;dact2-/-* mutants. Although at a very low frequency, ectopic expression of *capn8* mRNA recapitulated the *dact1-/-;dact2-/-* mutant craniofacial phenotype, suggesting that inhibition of *capn8* expression in the mesoderm by dact is required for normal morphogenesis. Genes involved in calcium ion binding were also differentially expressed in the *dact1-/-;dact2-/-* mutants and we predict that altering intracellular calcium handling in conjunction with *capn8* overexpression would increase the frequency of the recapitulated *dact1-/-;dact2-/-* mutant phenotype.

Capn8 is described as a stomach-specific calpain and a role during embryogenesis has not been previously described. Calpains are typically calcium-activated proteases and it is feasible that Capn8 is active in response to Wnt/Ca^{2+} signaling. A close family member, Capn2 has been found to modulate Wnt signaling by degradation of beta-catenin (Zanardelli, Christodoulou et al. 2013, Konze, van Diepen et al. 2014). These findings suggest that dact-dependent suppression of *capn8* expression is necessary for normal embryogenesis and craniofacial morphogenesis, further expanding the functional repertoire of *dact1/2*. Further research is required to examine the direct regulatory role of dacts on *capn8* expression. Our findings also warrant investigation into the role of *capn8* during embryogenesis and possible implications for known craniofacial or other disorders. Recently, Capn8 has been implicated in EMT associated with cancer metastasis (Zhong, Xu et al. 2022, Song, Cui et al. 2024) and Xenopus capn8 was found to be required for CNCC migration (Cousin, Abbruzzese et al. 2011). Together these findings support a role of *capn8* in CNCC migration and craniofacial morphogenesis.

Another gene identified in our scRNA-seq data to be differentially expressed in the *dact1-/-,dact2-/-* but not the *gpc4-/-* embryos was *smad1*. Smad1 acts in the TGF-β signaling pathway and dact2 has been described to inhibit TGF-β and Nodal signaling by promoting the degradation of Nodal receptors (Zhang, Zhou et al. 2004, Su, Zhang et al. 2007, Meng, Cheng et al. 2008, Kivimae, Yang et al. 2011). Zebrafish Nodal pathway mutants (*cyc/ndr2*, *oep/tdgf1*, *sqt/ndr1*) exhibit medially displaced eyes (Hatta, Kimmel et al. 1991, Brand, Heisenberg et al. 1996, Heisenberg

and Nusslein-Volhard 1997, Feldman, Gates et al. 1998, Zhang, Talbot et al. 1998) and it is robustly feasible that dysregulation of TGF-β signaling in the *dact1*-/-;*dact2*-/-mutant contributes to the craniofacial phenotype. Future research will examine the role of *dact1* and *dact2* in the coordination of Wnt and TGF-β signaling and the importance of this coordination in the context of craniofacial development. Of note, Sonic Hedgehog (shh) signaling is a principal regulator to the vertebrate midline (Chiang, Litingtung et al. 1996, Ribes, Balaskas et al. 2010), and important in the development of the zebrafish floorplate (Halpern, Hatta et al. 1997, Odenthal, van Eeden et al. 2000). Mutants with disrupted *shh* expression or signaling (*cyc/ndr2*, *smo*, *oep/tdgf1*) exhibit medially displaced eyes similar to the *dact1/2* mutants (Brand, Heisenberg et al. 1996, Chen, Burgess et al. 2001). We did not find any genes within the sonic hedgehog pathway to be differentially expressed in *dact1/2* mutants, though post-transcriptional interactions cannot be ruled out.

This study has uncovered the genetic requirement of *dact1* and *dact2* in embryonic CE and craniofacial morphogenesis, delineated the genetic interaction with Wnt genes and identified capn8 as a modifier of this process. As with most studies, this work has contributed some new knowledge but generated more questions than answers. Future work will delineate the molecular differences across the different *dact1/2* and other Wnt mutants to further identify determinants of craniofacial morphogenesis; and to connect these findings to clinically important Wnt regulators of facial morphology and pathology.

Methods

Animals and CRISPR/Cas9 targeted mutagenesis

All animal husbandry and experiments were performed in accordance with approval from Massachusetts General Hospital Institutional Animal Care and Usage Committee. Zebrafish (*Danio rerio*) embryos and adults were maintained in accordance with institutional protocols. Embryos were raised at 28.5 C in E3 medium (Carroll, Macias Trevino et al. 2020) and staged visually and according to standardized developmental timepoints (Westerfield 1993). All zebrafish lines used for experiments and gene editing were generated from the Tubingen or AB strain. The *wnt11f2* mutant line and gpc4-/-mutant line were obtained from Zebrafish International Resource Center (wnt11f2$^{tx226/+}$ and gpc4$^{hi1688Tg/+}$ respectively). The *wls*-/-mutant line was originally gifted to the lab and independently generated, as previously described (Rochard, Monica et al. 2016). The *sox10*:kaede transgenic line was previously generated and described by our lab (Dougherty, Kamel et al. 2012).

CRISPR sgRNA guides were designed using computational programs ZiFiT Targeter v4.2 (*zifit.partners.org/ZiFit*) (Sander, Zaback et al. 2007), *crispr.mit.edu* (*https://zlab.bio/guide-design-resouces*) (Ran, Hsu et al. 2013), and ChopChop (*https://chopchop.cbu.uib.no*) (Montague, Cruz et al. 2014) with traditional sequence constraints. Guides were chosen that were predicted to give high efficiency and specificity. Guides best meeting these parameters were selected in exon 2 of *dact1* and exon 4 of *dact2*. No suitable gRNA with sufficient efficiency were identified for *dact2* 5' of exon 4 and the resulting phenotype was reassuring compared to previous morpholino published results. Guides for *dact1* and *dact2* and Cas9 protein were prepared and microinjected into 1 cell-stage zebrafish embryos and founders were identified as previously described (Carroll, Macias Trevino et al. 2020). Primers flanking the sgRNA guide site were designed for genotyping and fragment analysis was performed on genomic DNA to detect base pair insertion/deletion. Sanger sequencing was performed to verify targeted gene mutation and confirm the inclusion of a premature stop codon. *dact1* forward primer: TACAGAAGCTGCTGAAGTACCG, *dact1* reverse primer:

CCCTCTCTCAAAGTGTTTTGGT, *dact2* forward primer:

TGAAGAGCTCCACTCCCCTGT, *dact2* reverse primer:

GCAGTTGAGGTCCATTCAGC.

RT qPCR analysis

Pooled wildtype, *dact1*-/-, *dact2*-/-, and *dact1/2*-/-fish were collected and RNA extractions were performed using RNeasy Mini Kit (Qiagen). cDNA was generated using High Capacity cDNA Reverse Transcription Kit (ThermoFisher). Quantitative PCR (qPCR) was performed using *dact1* (Dr03152516_m1) and *dact2* (Dr03426298_s1) TaqMan Gene Expression Assays. Expression was normalized to 18S rRNA expression (Hs03003631_g1). TaQman Fast Advanced master mix (ThermoFisher) and a StepOnePlus Real-Time PCR system (Applied Biosystems) were used to measure relative mRNA levels, which were calculated using the ddCT method.

Microinjection of mRNA

Template DNA for *in vivo* mRNA transcription was generated by PCR amplification of the gene of interest from a zebrafish embryo cDNA library and cloning into pCS2+8 destination plasmid. mRNA for injection was generated using an *in vitro* transcription kit (Invitrogen mMessage mMachine). One-cell stage zebrafish embryos were injected with 2 nL of mRNA in solution. To test genetic knockout specificity, 150 or 300 pg or dact1, dact2 or dact1 and dact2 mRNA was injected. For capn8 overexpression analysis 200 pg or GFP or capn8 mRNA was injected. Following phenotyping analysis, genotypes were determined by fragment analysis of *dact1* and *dact2* genotyping PCR products.

Wholemount and RNAscope *in situ* hybridization

Wholemount *in situ* hybridization was performed as previously described (Carroll, Macias Trevino et al. 2020). Zebrafish embryonic cDNA was used as a template to generate riboprobes. Primers were designed to PCR amplify the specific riboprobe sequence with a T7 promoter sequence linked to the reverse primer. *In vitro* transcription was performed using a T7 polymerase and DIG-labeled nucleotides (Roche). RNAscope was performed on sectioned zebrafish larvae as previously described (Carroll, Macias Trevino et al. 2020). Probes were designed by and purchased from ACD Bio. Hybridization and detection was performed according to the manufacturer's protocol. Sections were imaged using a confocal microscope (Leica SP8) and z-stack maximum projections were generated using Fiji software.

Alcian blue staining and imaging

Alcian blue staining and imaging were performed at 4 dpf as previously described (Carroll, Macias Trevino et al. 2020). Briefly, larvae were fixed in 4% v/v formaldehyde overnight at 4°C. Larvae were dehydrated in 50% v/v ethanol and stained with Alcian blue as described (Walker and Kimmel 2007). Whole and dissected larvae were imaged in 3% w/v methylcellulose using a Nikon Eclipse 80i compound microscope with a Nikon DS Ri1 camera. Z-stacked images were taken and extended depth of field was calculated using NIS Element BR 3.2 software. Images were processed using Fiji software. After image capture embryos were genotyped by PCR and fragment analysis.

Axis measurements

Compound *dact1*+/-; *dact2*+/- zebrafish were in-crossed and progeny were collected from 2 separate clutches and fixed in 4% formaldehyde at approximately 8ss. Embryos were individually imaged using a Zeiss Axiozoom stereoscope and processed for DNA extraction and genotyping (Westerfield 1993). Images were analyzed using Fiji. A circle was drawn to overlay the yolk and the geometric center was determined using the function on Fiji. Using the Fiji angle tool, lines were drawn from the center point to the anterior-most point of the embryo and from the center to

the posterior-most point of the embryo. The resulting inner angle of these lines was determined. Each angle measurement was then calculated as a ratio to the average angle of the wildtype embryos. ANOVA was performed to determine statistical significance, $p<0.05$.

Lineage analysis of CNCCs

Live embryos were mounted in 1% w/v low melt agarose and covered with E3 medium containing 0.013% w/v tricaine. Wildtype control and dact1/2-/-compound mutants on a Tg(*soxI0*:kaede) background were imaged on a Leica DMi8 confocal microscope and photoconverted using the UV laser (404 nm) until the green kaede fluorescence disappeared. For each embryo, one side was photoconverted and the contralateral side served as an internal control. After photoconversion, embryos were removed from the agarose and raised in E3 medium at 28.5 C until the required developmental timepoint, at which time they were similarly re-mounted and re-imaged. Z-stacked images were processed as maximum-intensity projections using Fiji software.

Single-cell RNA sequencing (scRNA-seq)

Single-cell transcriptomic analyses were performed on 10 zebrafish embryos, including 4 wildtype, 3 *dact1-/-;dact2-/-* compound mutant, and 3 *gpc4-/-* mutant embryos. Embryos were collected at 4 ss with *dact1-/-,dact2-/-* and *gpc4-/-* mutants being identified by their truncated body axis. Embryos were dechorionated with a short (approximately 10 min) incubation in 1 mg/ml Pronase and then washed 3x in embryo medium. Cell dissociation was performed with modifications as previously described (Farrell, Wang et al. 2018). Each embryo was transferred to 50 µl DMEM/F12 media on ice. To dissociate cells, media was replaced with 200 µl DPBS (without Ca^{2+} and Mg^{2+}) with 0.1% w/v BSA. Embryos were disrupted by pipetting 10x with a P200 pipette tip. 500 µl of DPBS + 0.1% BSA was added and cells were centrifuged at 300xg for 1 min. Cell pellets were resuspended in 200 µl DPBS + 0.1% BSA and kept on ice. Just prior to encapsulation, cells were passed through a 40 µm cell strainer, and cell counts and viability were measured. After droplet encapsulation, barcoding, and library preparation using the 10X Genomics Chromium Single Cell 3' kit (version 3), data were sequenced on an Illumina NovaSeq 6000 sequencer.

FASTQ files were demultiplexed and aligned to the GRCz11 build of the zebrafish genome using Cellranger (version 6.1.0) (Zheng, Terry et al. 2017). Raw Cellranger count matrices were imported into R (version 4.1.2) using Seurat (version 4.1.0) (Hao, Hao et al. 2021). First, we reviewed data for quality and excluded any droplet that did not meet all of the following criteria: (i) have at least 1,500 unique molecular identifiers (UMIs), (ii) covering at least 750 distinct genes; (iii) have <5% of genes mapping to the mitochondrial genome; and (iv) have a log10 of detected genes per UMI >80%. After quality control, the dataset was also filtered to exclude genes with a detection rate below 1 in 3,000 cells, leaving a total of 20,078 distinct genes expressed across 19,457 cells for analysis.

The quality-controlled count data were normalized using Pearson's residuals from the regularized negative binomial regression model, as implemented in Seurat::SCTransfrom (Hafemeister and Satija 2019). When computing the SCT model, the effect of the total number of UMIs and number of detected genes per cell were regressed out. After normalization, the top 3,000 most variably expressed genes were used to calculate principal components (PCs). Data were then integrated by source sample using Harmony (version 0.1.0) (Korsunsky, Millard et al. 2019). A two-dimensional uniform manifold approximation and projection (UMAP) (Becht, McInnes et al. 2018) was then derived from the first 40 Harmony embeddings for visualization. Using the integrated Harmony embeddings, clusters were defined with the Louvain clustering method, as implemented within Seurat. A resolution of 0.3 was used for cluster definition. Cluster identities were assigned by manually reviewing the results of Seurat::FindAllMarkers, searching for genes associated to known developmental lineages. Gene expression data for key markers that guided cluster identity assignment were visualized using Seurat::DotPlot.

Following this detailed annotation, some clusters were grouped to focus downstream analyses on 3 major lineages: ventral mesoderm (grouping cells from the pronephros, vasculogenic/myeloid precursors, hematopoietic cells, heart primordium, and cephalic mesoderm clusters), dorsal mesoderm (adaxial cells, segmental plate, and paraxial mesoderm), ectoderm (CNS, mid/hindbrain boundary, spinal cord, and neural crest). For those 3 lineages, single-cell level data were aggregated per sample and cluster to perform pseudobulk differential expression analyses (DEA) contrasting genotypes. Independent pairwise comparisons of *dact1-/-;dact2-/-* versus wildtype and *gpc4-/-* vs wildtype were performed using DESeq2 (v1.34.0) (Love, Huber et al. 2014). P-values were corrected for multiple testing using the default Benjamini-Hochberg method; log2 fold change values were corrected using the apeglm shrinkage estimator (Zhu, Ibrahim et al. 2019). Significance was defined as an adjusted p-value < 0.1 and log2 fold change > 0.58 in absolute value. Heatmaps of the top most significant differentially expressed genes were generated from the regularized log transformed data using pheatmap (version 1.0.12). Overlap in significant genes across pairwise comparisons were determined and visualized in Venn diagrams. Over-representation analyses against the Gene Ontology (GO) database were ran using clusterProfiler (version 4.2.2) (Wu, Hu et al. 2021), using as input the set of genes found to be differentially expressed in the comparison of *dact1-/-;dact-/-* versus wildtype but not *gpc4-/-* versus wildtype. Sequencing data have been deposited in GEO under accession code GSE240264.

Statistical analysis

Analyses were performed using Prism Software (GraphPad) unless otherwise specified. An unpaired Student's *t* test or one-way ANOVA with multiple comparisons was used as indicated and a *P*-value <0.05 was considered significant. Graphs represent the mean +/-the SEM and *n* represents biological replicates. For categorical data (normal vs. mutant phenotype) a Fisher exact test was performed between *gfp* and *capn8* injected embryos and the odds ratio was determined. The confidence interval was determined by the Baptista-Pike method.

Acknowledgements

We thank Christoph Seiler, Adele Donohue and the Aqautics Facility team at Children's Hospital of Philadelphia; and Jessica Bethoney at Massachusetts General Hospital (MGH) for their excellent management of our zebrafish colonies and facilities. We thank the MGH Next Generation Sequencing Core for cell encapsulation, cDNA library preparation, and sequencing. Single-cell sequencing analysis was performed by the Harvard Chan Bioinformatics Core. Work by A.J. was funded in part by the Harvard Stem Cell Institute. We appreciate and acknowledge the generous funding support from the Shriners Hospitals for Children. This work was supported by the National Institutes of Health grant R01DE027983 to E.C.L.

Supplemental Figures

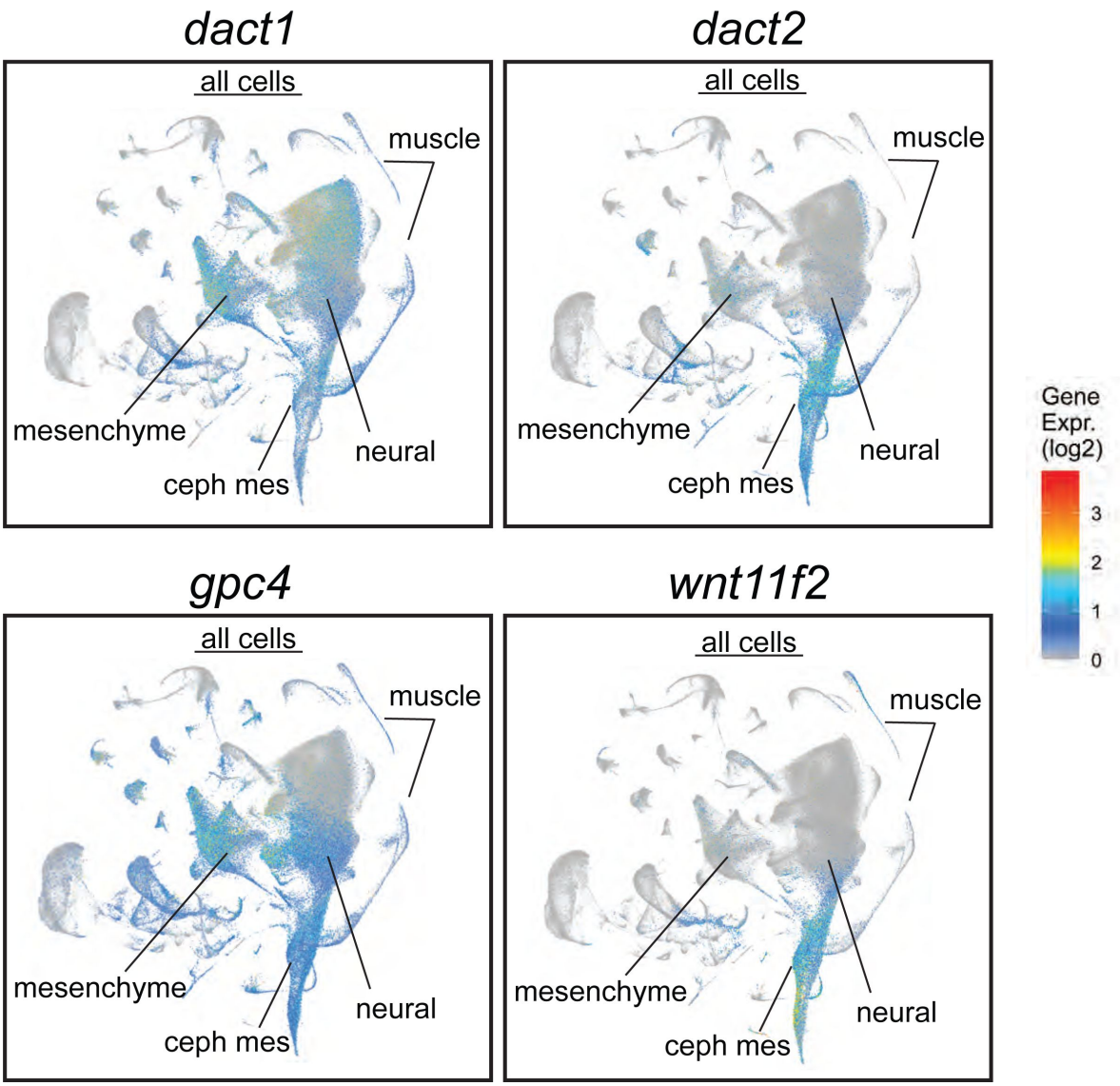


Figure S1.

Daniocell single-cell RNAseq analysis with a display of *dact1*, *dact2*, *gpc4,* and *wnt11f2* in all cell clusters from 3-120 hpf of development (Farrell, Wang et al. 2018). Cephalic mesoderm (ceph mes), mesenchyme, neural ectoderm, and muscle clusters are noted.

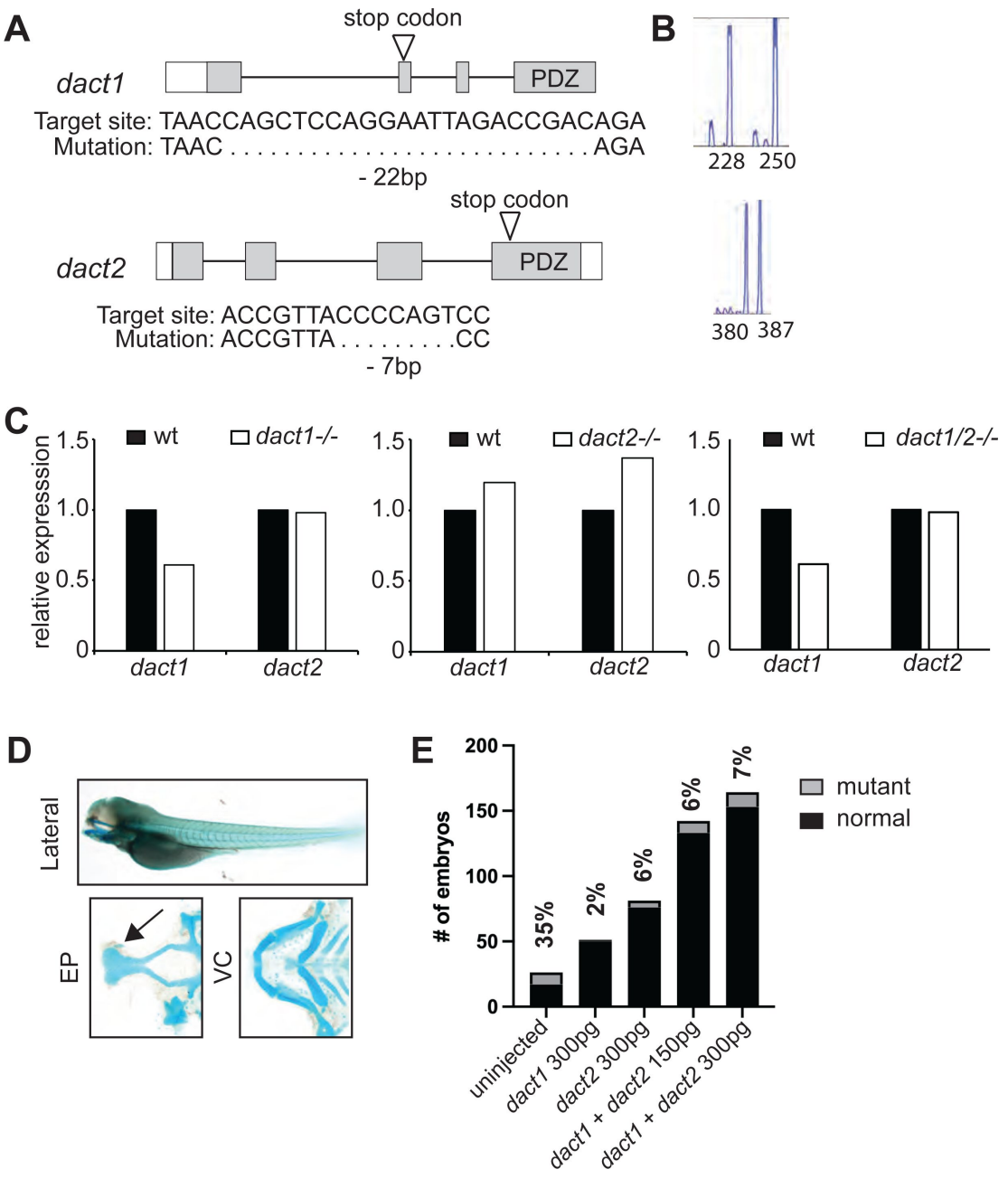


Figure S2.

Characterization of CRISPR/Cas9 generated *dact1-/-* and *dact2-/-* mutants. **A)** Schematic representations of *dact1* and *dact2* exons, positions of guide RNA target site, introduced premature stop codon (arrow), and sequences of mutations. **B)** DNA fragment analysis of *dact1+/-* and *dact2+/-* animals showing wildtype (250 and 387 bp respectively and mutant (228 and 380 bp respectively) **C)** Expression levels of *dact1* and *dact2* mRNA by RT-qPCR in 12 hpf *dact1-/-* mutants, *dact2-/-* mutants, and *dact1/2-/-* compound mutants. 8 embryos were pooled for mRNA isolation per sample. **D)** Injection of *dact1* mRNA, *dact2* mRNA, or a combination of *dact1* and *dact2* mRNA rescues the rod-shaped ethmoid plate phenotype in *dact1/2-/-* compound mutants. Representative images of Alcian blue stained *dact1/2-/-* double mutant treated with 300 pg *dact1* mRNA and 300 pg *dact2* mRNA. Arrow highlights normal ethmoid plate (EP). Visceral cartilage (VC) also appeared normal. **E)** Quantification of the mutant craniofacial phenotype observed in a *dact1-/-,dact2+/-* breeding in-cross. Without mRNA injection, the mutant phenotype was observed at approximately (35%) the expected Mendelian ratio of 25%. Injection with *dact1* mRNA, *dact2* mRNA, or a combination of *dact1* and *dact2* mRNA decreased the frequency that the mutant craniofacial phenotype was observed.

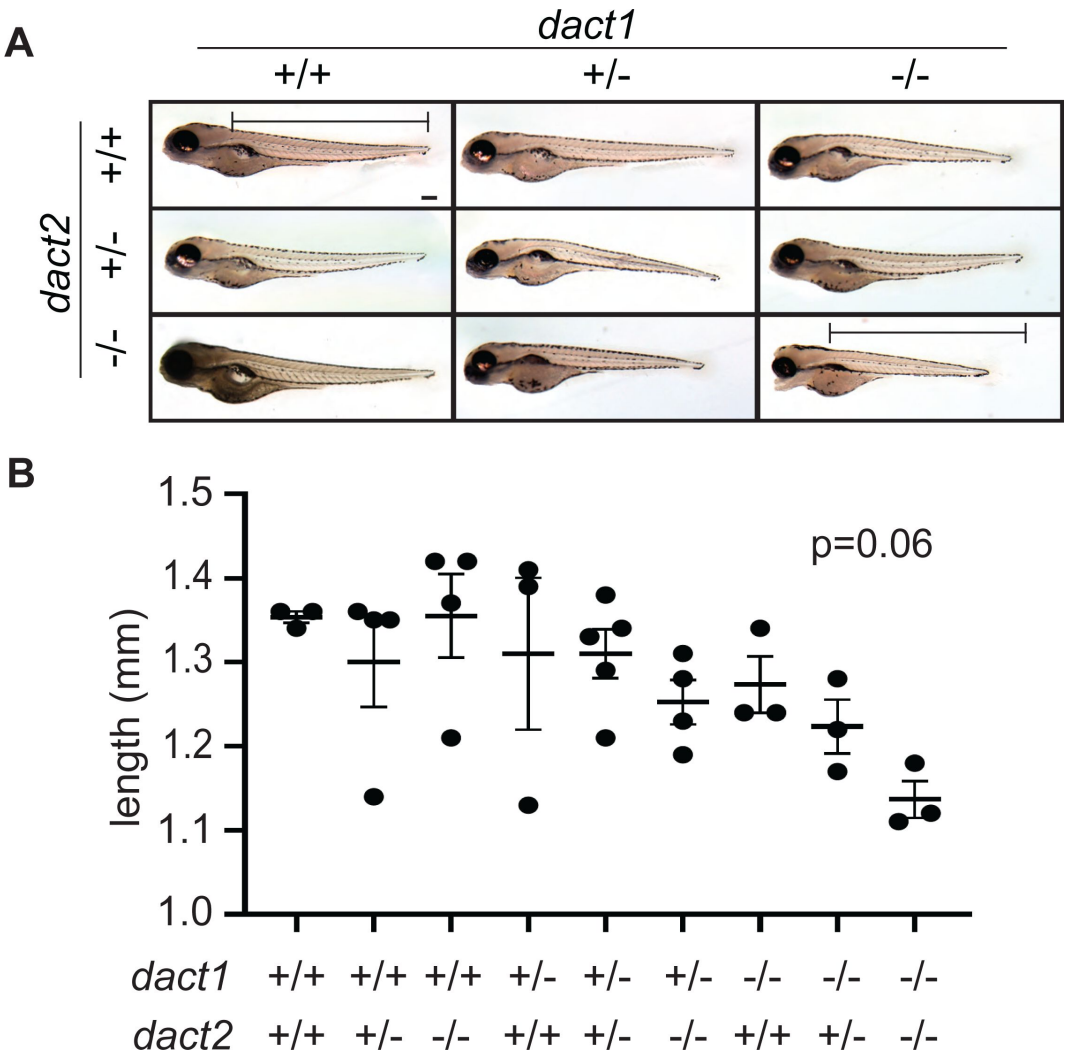


Figure S3.

Loss of *dact1* and *dact2* tends to decrease total body length. **A)** Representative brightfield images of 4 dpf larvae of compound *dact1* and *dact2* mutant genotypes. Bar represents *dact1*+/+,*dact2*+/+ body length measurement. Scale bar: 100 μm. **B)** Scatter plot of body length measurement of compound *dact1* and *dact2* mutant genotypes at 4 dpf. ANOVA p=0.06. n=3-4.

Figure S4.

Cluster abundance across genotype groups. Scatter box plots showing the frequency of each identified cell cluster between *dact1/2-/-*, *gpc4-/-*, and wildtype samples.

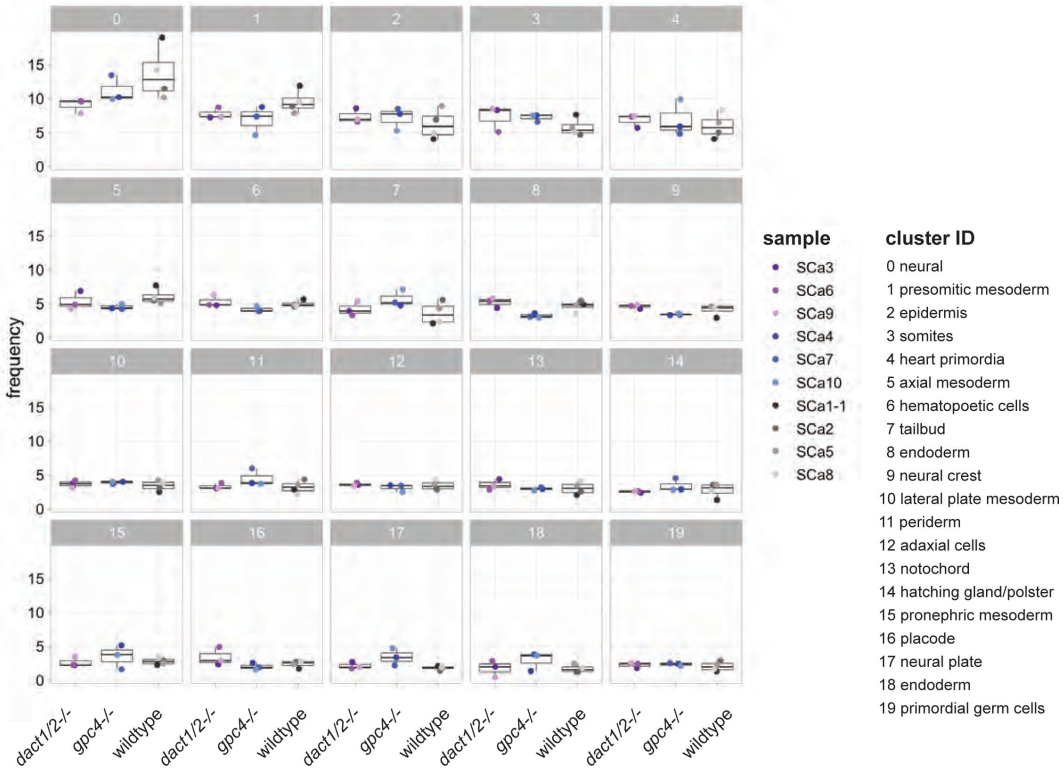
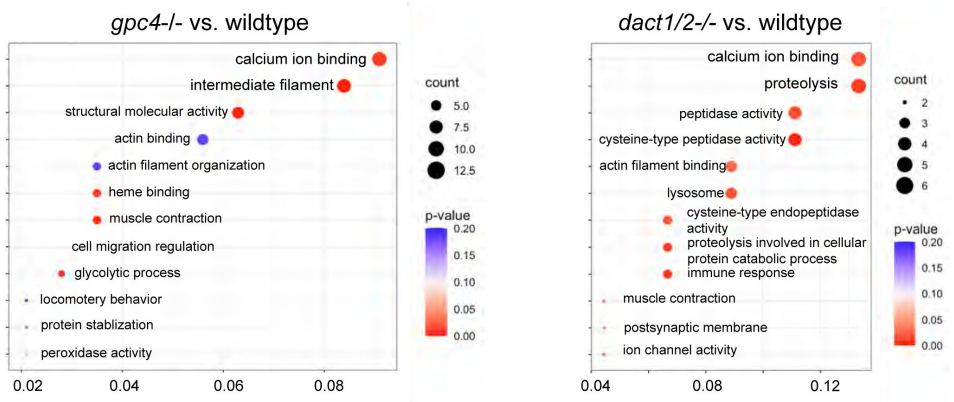


Figure S5.

Loss of *gpc4* and loss of *dact1/2* leads to distinct changes in gene expression profiles but with some overlapping functions. GO analysis of DEGs identified between *gpc4-/-*and wildtype embryos and *dact1/2-/-*and wildtype embryos found changes in calcium ion binding and actin interaction in both mutants.



References

1. Alhazmi N. *et al.* (2021) **Synergistic roles of Wnt modulators R-spondin2 and R-spondin3 in craniofacial morphogenesis and dental development** *Sci Rep* **11**

2. Banziger C., Soldini D., Schutt C., Zipperlen P., Hausmann G., Basler K. (2006) **Wntless, a conserved membrane protein dedicated to the secretion of Wnt proteins from signaling cells** *Cell* **125**:509–522

3. Bartscherer K., Pelte N., Ingelfinger D., Boutros M. (2006) **Secretion of Wnt ligands requires Evi, a conserved transmembrane protein** *Cell* **125**:523–533

4. Becht E., McInnes L., Healy J., Dutertre C. A., Kwok I. W. H., Ng L. G., Ginhoux F., Newell E. W. (2018) **Dimensionality reduction for visualizing single-cell data using UMAP** *Nat Biotechnol*

5. Bradford Y. M. *et al.* (2022) **Zebrafish information network, the knowledgebase for Danio rerio research** *Genetics* **220**

6. Brand M. *et al.* (1996) **Mutations affecting development of the midline and general body shape during zebrafish embryogenesis** *Development* **123**:129–142

8. Brott B. K., Sokol S. Y. (2005) **Frodo proteins: modulators of Wnt signaling in vertebrate development** *Differentiation* **73**:323–329

9. Carroll S. H., Macias Trevino C., Li E. B., Kawasaki K., Myers N., Hallett S. A., Alhazmi N., Cotney J., Carstens R. P., Liao E. C. (2020) **An Irf6-Esrp1/2 regulatory axis controls midface morphogenesis in vertebrates** *Development* **147**

10. Chen W., Burgess S., Hopkins N. (2001) **Analysis of the zebrafish smoothened mutant reveals conserved and divergent functions of hedgehog activity** *Development* **128**:2385–2396

11. Cheyette B. N., Waxman J. S., Miller J. R., Takemaru K., Sheldahl L. C., Khlebtsova N., Fox E. P., Earnest T., Moon R. T. (2002) **Dapper, a Dishevelled-associated antagonist of beta-catenin and JNK signaling, is required for notochord formation** *Dev Cell* **2**:449–461

12. Chiang C., Litingtung Y., Lee E., Young K. E., Corden J. L., Westphal H., Beachy P. A. (1996) **Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function** *Nature* **383**:407–413

13. Clevers H., Nusse R. (2012) **Wnt/beta-catenin signaling and disease** *Cell* **149**:1192–1205

14. Corey D. R., Abrams J. M. (2001) **Morpholino antisense oligonucleotides: tools for investigating vertebrate development** *Genome Biol* **2**

15. Cousin H., Abbruzzese G., Kerdavid E., Gaultier A., Alfandari D. (2011) **Translocation of the cytoplasmic domain of ADAM13 to the nucleus is essential for Calpain8-a expression and cranial neural crest cell migration** *Dev Cell* **20**:256–263

16. Dougherty M., Kamel G., Shubinets V., Hickey G., Grimaldi M., Liao E. C. (2012) **Embryonic fate map of first pharyngeal arch structures in the sox10: kaede zebrafish transgenic model** *J Craniofac Surg* **23**:1333–1337

17. Dutton J. R., Antonellis A., Carney T. J., Rodrigues F. S., Pavan W. J., Ward A., Kelsh R. N. (2008) **An evolutionarily conserved intronic region controls the spatiotemporal expression of the transcription factor Sox10** *BMC Dev Biol* **8**

18. Farnsworth D. R., Saunders L. M., Miller A. C. (2020) **A single-cell transcriptome atlas for zebrafish development** *Dev Biol* **459**:100–108

19. Farrell J. A., Wang Y., Riesenfeld S. J., Shekhar K., Regev A., Schier A. F. (2018) **Single-cell reconstruction of developmental trajectories during zebrafish embryogenesis** *Science* **360**

20. Feldman B., Gates M. A., Egan E. S., Dougan S. T., Rennebeck G., Sirotkin H. I., Schier A. F., Talbot W. S. (1998) **Zebrafish organizer development and germ-layer formation require nodal-related signals** *Nature* **395**:181–185

21. Gao X., Wen J., Zhang L., Li X., Ning Y., Meng A., Chen Y. G. (2008) **Dapper1 is a nucleocytoplasmic shuttling protein that negatively modulates Wnt signaling in the nucleus** *J Biol Chem* **283**:35679–35688

22. Gillhouse M., Wagner Nyholm M., Hikasa H., Sokol S. Y., Grinblat Y. (2004) **Two Frodo/Dapper homologs are expressed in the developing brain and mesoderm of zebrafish** *Dev Dyn* **230**:403–409

23. Gloy J., Hikasa H., Sokol S. Y. (2002) **Frodo interacts with Dishevelled to transduce Wnt signals** *Nat Cell Biol* **4**:351–357

24. Hafemeister C., Satija R. (2019) **Normalization and variance stabilization of single-cell RNA-seq data using regularized negative binomial regression** *Genome Biol* **20**

25. Halpern M. E., Hatta K., Amacher S. L., Talbot W. S., Yan Y. L., Thisse B., Thisse C., Postlethwait J. H., Kimmel C. B. (1997) **Genetic interactions in zebrafish midline development** *Dev Biol* **187**:154–170

26. Hammerschmidt M. *et al.* (1996) **Mutations affecting morphogenesis during gastrulation and tail formation in the zebrafish, Danio rerio** *Development* **123**:143–151

27. Hao Y. *et al.* (2021) **Integrated analysis of multimodal single-cell data** *Cell* **184**:3573–3587

28. Hashimoto M., Morita H., Ueno N. (2014) **Molecular and cellular mechanisms of development underlying congenital diseases** *Congenit Anom (Kyoto* **54**:1–7

29. Hatta K., Kimmel C. B., Ho R. K., Walker C. (1991) **The cyclops mutation blocks specification of the floor plate of the zebrafish central nervous system** *Nature* **350**:339–341

30. Heasman J (2002) **Morpholino oligos: making sense of antisense?** *Dev Biol* **243**:209–214

31. Heisenberg C. P. *et al.* (1996) **Genes involved in forebrain development in the zebrafish, Danio rerio** *Development* **123**:191–203

32. Heisenberg C. P., Nusslein-Volhard C. (1997) **The function of silberblick in the positioning of the eye anlage in the zebrafish embryo** *Dev Biol* **184**:85–94

33. Heisenberg C. P., Tada M., Rauch G. J., Saude L., Concha M. L., Geisler R., Stemple D. L., Smith J. C., Wilson S. W. (2000) **Silberblick/Wnt11 mediates convergent extension movements during zebrafish gastrulation** *Nature* **405**:76–81

34. Hikasa H., Sokol S. Y. (2004) **The involvement of Frodo in TCF-dependent signaling and neural tissue development** *Development* **131**:4725–4734

35. Hunter N. L., Hikasa H., Dymecki S. M., Sokol S. Y. (2006) **Vertebrate homologues of Frodo are dynamically expressed during embryonic development in tissues undergoing extensive morphogenetic movements** *Dev Dyn* **235**:279–284

36. Huybrechts Y., Mortier G., Boudin E., Van Hul W. (2020) **WNT Signaling and Bone: Lessons From Skeletal Dysplasias and Disorders** *Front Endocrinol (Lausanne* **11**

37. Ji Y., Hao H., Reynolds K., McMahon M., Zhou C. J. (2019) **Wnt Signaling in Neural Crest Ontogenesis and Oncogenesis** *Cells* **8**

38. Kague E., Gallagher M., Burke S., Parsons M., Franz-Odendaal T., Fisher S. (2012) **Skeletogenic fate of zebrafish cranial and trunk neural crest** *PLoS One* **7**

39. Kamel G., Hoyos T., Rochard L., Dougherty M., Kong Y., Tse W., Shubinets V., Grimaldi M., Liao E. C. (2013) **Requirement for frzb and fzd7a in cranial neural crest convergence and extension mechanisms during zebrafish palate and jaw morphogenesis** *Dev Biol* **381**:423–433

40. Kettunen P., Kivimae S., Keshari P., Klein O. D., Cheyette B. N., Luukko K. (2010) **Dact1-3 mRNAs exhibit distinct expression domains during tooth development** *Gene Expr Patterns* **10**:140–143

41. Kim D. H., Kim E. J., Kim D. H., Park S. W. (2020) **Dact2 is involved in the regulation of epithelial-mesenchymal transition** *Biochem Biophys Res Commun* **524**:190–197

42. Kimmel C. B., Miller C. T., Moens C. B. (2001) **Specification and morphogenesis of the zebrafish larval head skeleton** *Dev Biol* **233**:239–257

43. Kivimae S., Yang X. Y., Cheyette B. N. (2011) **All Dact (Dapper/Frodo) scaffold proteins dimerize and exhibit conserved interactions with Vangl, Dvl, and serine/threonine kinases** *BMC Biochem* **12**

44. Kok F. O. *et al.* (2015) **Reverse genetic screening reveals poor correlation between morpholino-induced and mutant phenotypes in zebrafish** *Dev Cell* **32**:97–108

45. Konze S. A., van Diepen L., Schroder A., Olmer R., Moller H., Pich A., Weissmann R., Kuss A. W., Zweigerdt R., Buettner F. F. (2014) **Cleavage of E-cadherin and beta-catenin by calpain affects Wnt signaling and spheroid formation in suspension cultures of human pluripotent stem cells** *Mol Cell Proteomics* **13**:990–1007

46. Korsunsky I., Millard N., Fan J., Slowikowski K., Zhang F., Wei K., Baglaenko Y., Brenner M., Loh P. R., Raychaudhuri S. (2019) **Fast, sensitive and accurate integration of single-cell data with Harmony** *Nat Methods* **16**:1289–1296

47. Lee H., Cheong S. M., Han W., Koo Y., Jo S. B., Cho G. S., Yang J. S., Kim S., Han J. K. (2018) **Head formation requires Dishevelled degradation that is mediated by March2 in concert with Dapper1** *Development* **145**

48. Li X., Florez S., Wang J., Cao H., Amendt B. A. (2013) **Dact2 represses PITX2 transcriptional activation and cell proliferation through Wnt/beta-catenin signaling during odontogenesis** *PLoS One* **8**

49. Ling I. T., Rochard L., Liao E. C. (2017) **Distinct requirements of wls, wnt9a, wnt5b and gpc4 in regulating chondrocyte maturation and timing of endochondral ossification** *Dev Biol* **421**:219–232

50. Logan C. Y., Nusse R. (2004) **The Wnt signaling pathway in development and disease** *Annu Rev Cell Dev Biol* **20**:781–810

51. Loh K. M., van Amerongen R., Nusse R. (2016) **Generating Cellular Diversity and Spatial Form: Wnt Signaling and the Evolution of Multicellular Animals** *Dev Cell* **38**:643–655

52. Love M. I., Huber W., Anders S. (2014) **Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2** *Genome Biol* **15**

53. Ma B., Liu B., Cao W., Gao C., Qi Z., Ning Y., Chen Y. G. (2015) **The Wnt Signaling Antagonist Dapper1 Accelerates Dishevelled2 Degradation via Promoting Its Ubiquitination and Aggregate-induced Autophagy** *J Biol Chem* **290**:12346–12354

54. Macqueen D. J., Wilcox A. H. (2014) **Characterization of the definitive classical calpain family of vertebrates using phylogenetic, evolutionary and expression analyses** *Open Biol* **4**

55. Mehta S., Hingole S., Chaudhary V. (2021) **The Emerging Mechanisms of Wnt Secretion and Signaling in Development** *Front Cell Dev Biol* **9**

56. Meng F., Cheng X., Yang L., Hou N., Yang X., Meng A. (2008) **Accelerated re-epithelialization in Dpr2-deficient mice is associated with enhanced response to TGFbeta signaling** *J Cell Sci* **121**:2904–2912

57. Montague T. G., Cruz J. M., Gagnon J. A., Church G. M., Valen E. (2014) **CHOPCHOP: a CRISPR/Cas9 and TALEN web tool for genome editing** *Nucleic Acids Res* **42**:W401–407

58. Morcos P. A., Vincent A. C., Moulton J. D. (2015) **Gene Editing Versus Morphants** *Zebrafish* **12**

59. Mork L., Crump G. (2015) **Zebrafish Craniofacial Development: A Window into Early Patterning** *Curr Top Dev Biol* **115**:235–269

60. Muyskens J. B., Kimmel C. B. (2007) **Tbx16 cooperates with Wnt11 in assembling the zebrafish organizer** *Mech Dev* **124**:35–42

61. Nasevicius A., Ekker S. C. (2000) **Effective targeted gene 'knockdown' in zebrafish** *Nat Genet* **26**:216–220

62. Niehrs C (2012) **The complex world of WNT receptor signalling** *Nat Rev Mol Cell Biol* **13**:767–779

63. Odenthal J., van Eeden F. J., Haffter P., Ingham P. W., Nusslein-Volhard C. (2000) **Two distinct cell populations in the floor plate of the zebrafish are induced by different pathways** *Dev Biol* **219**:350–363

64. Oteiza P. *et al.* (2010) **Planar cell polarity signalling regulates cell adhesion properties in progenitors of the zebrafish laterality organ** *Development* **137**:3459–3468

65. Petersen C. P., Reddien P. W. (2009) **Wnt signaling and the polarity of the primary body axis** *Cell* **139**:1056–1068

66. Piotrowski T. *et al.* (1996) **Jaw and branchial arch mutants in zebrafish II: anterior arches and cartilage differentiation** *Development* **123**:345–356

67. Rabadan M. A., Herrera A., Fanlo L., Usieto S., Carmona-Fontaine C., Barriga E. H., Mayor R., Pons S., Marti E. (2016) **Delamination of neural crest cells requires transient and reversible Wnt inhibition mediated by Dact1/2** *Development* **143**:2194–2205

68. Ran F. A., Hsu P. D., Wright J., Agarwala V., Scott D. A., Zhang F. (2013) **Genome engineering using the CRISPR-Cas9 system** *Nat Protoc* **8**:2281–2308

69. Reynolds K., Kumari P., Sepulveda Rincon L., Gu R., Ji Y., Kumar S., Zhou C. J. (2019) **Wnt signaling in orofacial clefts: crosstalk, pathogenesis and models** *Dis Model Mech* **12**

70. Ribes V. *et al.* (2010) **Distinct Sonic Hedgehog signaling dynamics specify floor plate and ventral neuronal progenitors in the vertebrate neural tube** *Genes Dev* **24**:1186–1200

71. Rochard L., Monica S. D., Ling I. T., Kong Y., Roberson S., Harland R., Halpern M., Liao E. C. (2016) **Roles of Wnt pathway genes wls, wnt9a, wnt5b, frzb and gpc4 in regulating convergent-extension during zebrafish palate morphogenesis** *Development* **143**:2541–2547

72. Rossi A., Kontarakis Z., Gerri C., Nolte H., Holper S., Kruger M., Stainier D. Y. (2015) **Genetic compensation induced by deleterious mutations but not gene knockdowns** *Nature* **524**:230–233

73. Sander J. D., Zaback P., Joung J. K., Voytas D. F., Dobbs D. (2007) **Zinc Finger Targeter (ZiFiT): an engineered zinc finger/target site design tool** *Nucleic Acids Res* **35**:W599–605

74. Schilling T. F., Le Pabic P. (2009) **Fishing for the signals that pattern the face** *J Biol* **8**

75. Schubert F. R., Sobreira D. R., Janousek R. G., Alvares L. E., Dietrich S. (2014) **Dact genes are chordate specific regulators at the intersection of Wnt and Tgf-beta signaling pathways** *BMC Evol Biol* **14**

76. Shi D. L (2022) **Wnt/planar cell polarity signaling controls morphogenetic movements of gastrulation and neural tube closure** *Cell Mol Life Sci* **79**

77. Sisson B. E., Dale R. M., Mui S. R., Topczewska J. M., Topczewski J. (2015) **A role of glypican4 and wnt5b in chondrocyte stacking underlying craniofacial cartilage morphogenesis** *Mech Dev* **138**:279–290

78. Solnica-Krezel L. *et al.* (1996) **Mutations affecting cell fates and cellular rearrangements during gastrulation in zebrafish** *Development* **123**:67–80

79. Song N., Cui K., Zeng L., Fan Y., Wang Z., Shi P., Su W., Wang H. (2024) **Calpain 8 as a potential biomarker regulates the progression of pancreatic cancer via EMT and AKT/ERK pathway** *J Proteomics* **301**

80. Sorimachi H., Ishiura S., Suzuki K. (1993) **A novel tissue-specific calpain species expressed predominantly in the stomach comprises two alternative splicing products with and without Ca(2+)-binding domain** *J Biol Chem* **268**:19476–19482

81. Steinhart Z., Angers S. (2018) **Wnt signaling in development and tissue homeostasis** *Development* **145**

82. Su Y., Zhang L., Gao X., Meng F., Wen J., Zhou H., Meng A., Chen Y. G. (2007) **The evolutionally conserved activity of Dapper2 in antagonizing TGF-beta signaling** *FASEB J* **21**:682–690

83. Swartz M. E., Sheehan-Rooney K., Dixon M. J., Eberhart J. K. (2011) **Examination of a palatogenic gene program in zebrafish** *Dev Dyn* **240**:2204–2220

84. Tada M., Heisenberg C. P. (2012) **Convergent extension: using collective cell migration and cell intercalation to shape embryos** *Development* **139**:3897–3904

85. Thisse B. *et al.* (2001) **Thisse, B., Pflumio, S., Furthauer, M., Loppin, B., Heyer, V., Degrave, A., Woehl, R., Lux, A., Steffan, T., Charbonnier, X.Q., Thisse, C. (2001). "Expression of the zebrafish genome during embryogenesis." from http://zfin.org.**

86. Topczewski J., Sepich D. S., Myers D. C., Walker C., Amores A., Lele Z., Hammerschmidt M., Postlethwait J., Solnica-Krezel L. (2001) **The zebrafish glypican knypek controls cell polarity during gastrulation movements of convergent extension** *Dev Cell* **1**:251–264

87. Wada N., Javidan Y., Nelson S., Carney T. J., Kelsh R. N., Schilling T. F. (2005) **Hedgehog signaling is required for cranial neural crest morphogenesis and chondrogenesis at the midline in the zebrafish skull** *Development* **132**:3977–3988

88. Walker M. B., Kimmel C. B. (2007) **A two-color acid-free cartilage and bone stain for zebrafish larvae** *Biotech Histochem* **82**:23–28

89. Waxman J. S., Hocking A. M., Stoick C. L., Moon R. T. (2004) **Zebrafish Dapper1 and Dapper2 play distinct roles in Wnt-mediated developmental processes** *Development* **131**:5909–5921

90. Wen J., Chiang Y. J., Gao C., Xue H., Xu J., Ning Y., Hodes R. J., Gao X., Chen Y. G. (2010) **Loss of Dact1 disrupts planar cell polarity signaling by altering dishevelled activity and leads to posterior malformation in mice** *J Biol Chem* **285**:11023–11030

91. Westerfield M. (1993) **The zebrafish book: a guide for the laboratory use of zebrafish (Brachydanio rerio)**

92. Wiese K. E., Nusse R., van Amerongen R. (2018) **Wnt signalling: conquering complexity** *Development* **145**

93. Wong H. C., Bourdelas A., Krauss A., Lee H. J., Shao Y., Wu D., Mlodzik M., Shi D. L., Zheng J. (2003) **Direct binding of the PDZ domain of Dishevelled to a conserved internal sequence in the C-terminal region of Frizzled** *Mol Cell* **12**:1251–1260

94. Wu T. *et al.* (2021) **clusterProfiler 4.0: A universal enrichment tool for interpreting omics data** *Innovation (Camb* **2**

95. Zanardelli S., Christodoulou N., Skourides P. A. (2013) **Calpain2 protease: A new member of the Wnt/Ca(2+) pathway modulating convergent extension movements in Xenopus** *Dev Biol* **384**:83–100

96. Zhang J., Talbot W. S., Schier A. F. (1998) **Positional cloning identifies zebrafish one-eyed pinhead as a permissive EGF-related ligand required during gastrulation** *Cell* **92**:241–251

97. Zhang L., Gao X., Wen J., Ning Y., Chen Y. G. (2006) **Dapper 1 antagonizes Wnt signaling by promoting dishevelled degradation** *J Biol Chem* **281**:8607–8612

98. Zhang L., Zhou H., Su Y., Sun Z., Zhang H., Zhang L., Zhang Y., Ning Y., Chen Y. G., Meng A. (2004) **Zebrafish Dpr2 inhibits mesoderm induction by promoting degradation of nodal receptors** *Science* **306**:114–117

99. Zheng G. X. *et al.* (2017) **Massively parallel digital transcriptional profiling of single cells** *Nat Commun* **8**

100. Zhong X., Xu S., Wang Q., Peng L., Wang F., He T., Liu C., Ni S., He Z. (2022) **CAPN8 involves with exhausted, inflamed, and desert immune microenvironment to influence the metastasis of thyroid cancer** *Front Immunol* **13**

101. Zhu A., Ibrahim J. G., Love M. I. (2019) **Heavy-tailed prior distributions for sequence count data: removing the noise and preserving large differences** *Bioinformatics* **35**:2084–2092

Editors

Reviewer #1 (Public Review):

Summary:

This study explores the roles of dact1 and dact2 in zebrafish embryonic axis formation and craniofacial morphogenesis. The researchers aim to uncover the mechanisms by which dact1/2 modulates Wnt signaling during embryonic development and patterning. They propose distinct spatiotemporal roles for Dact1 and Dact2 proteins in zebrafish embryonic development, particularly their involvement in modulating noncanonical Wnt signaling during convergent extension events. The findings demonstrate that dact1 and dact2 have unique spatiotemporal expression domains during development and that mutations in dact1/2 lead to convergent extension defects. Furthermore, the study attempts to link these defects to craniofacial abnormalities resulting from dact1/2 mutations. Compound mutants were used to investigate the connection between dact1 and dact2, as single mutants did not exhibit craniofacial phenotypes. The research also includes comprehensive transcriptomics and pathway analyses of differentially expressed genes in dact1/2 mutants, revealing the overexpression of calpain 8, a calcium-dependent cysteine protease. The study suggests that the upregulation of calpain 8 is linked to the observed craniofacial dysmorphology in dact1/2 mutants, implying a potential connection between calpain 8 expression and craniofacial abnormalities.

Strengths:

• The study effectively recapitulates previous findings on the role of dact1/2 in modulating convergent extension during zebrafish embryogenesis.
• A combination of multiple approaches, including in vivo time-lapse imaging, is used to elucidate the etiology of the rod-like neurocranial phenotype in dact1/2 double mutants.
• The study utilizes both traditional and newly created mutant lines, analyzing them through single-cell transcriptomics.

Weaknesses:

(1) The authors successfully addressed reviewers' suggestions with revised experiments and explanations. However, the overall narrative struggles to build a more coherent storyline.
(2) The potential activity of truncated and upregulated dact mRNAs (Fig S2) and partially functional dact proteins needs further clarification.
(3) Data-rich figures, specifically Figs 6, 7, and 8D, could be simplified for better clarity.

https://doi.org/10.7554/eLife.91648.2.sa3

Reviewer #2 (Public Review):

Summary:

Non-canonical Wnt signaling plays an important role in morphogenesis, but how different components of the pathway are required to regulate different developmental events remains an open question. This paper focuses on elucidating the overlapping and distinct functions of dact1 and dact2, two Dishevelled-binding scaffold proteins, during zebrafish axis elongation and craniofacial development. By combining genetic studies, detailed phenotypic analysis, lineage tracing, and single cell RNA-sequencing, the authors aimed to understand (1) the relative function of dact1/2 in promoting axis elongation, (2) their ability to modulate phenotypes caused by mutations in other non-canonical wnt components, and (3) pathways downstream of dact1/2.
Corroborating previous findings, this paper showed that dact1/2 is required for convergent extension during gastrulation and body axis elongation. Strong qualitative evidence was also provided to support dact1/2's role in genetically modulating non-canonical wnt signaling to regulate body axis elongation and the morphology of the ethmoid plate (EP). However, the spatiotemporal function of dact1/2 remains unknown. The use of scRNA-seq identified novel pathways and targets downstream of dact1/2. Calpain 8 is one such example, and its overexpression in some of the dact1/2+/- embryos was able to phenocopy the dact1/2-/- mutant EP morphology, pointing to its sufficiency in driving the EP phenotype in a few embryos. However, the same effect was not observed in dact1-/-; dact2+/- embryos, leading to the question of how significant calpain 8 really is in this context. The requirement of calpain 8 in mediating the phenotype is unclear as well. This is the most novel aspect of the paper, but some weaknesses remain in convincingly demonstrating the importance of calpain 8.

Strengths:

(1) The generation of dact1/2 germline mutants and the use of genetic approaches to dissect their genetic interactions with wnt11f2 and gpc4 provide unambiguous and consistent results that inform the relative functions of dact1 and dact2, as well as their combined effects.
(2) Because the ethmoid plate exhibits a spectrum of phenotypes in different wnt genetic mutants, it is a useful system for studying how tissue morphology can be modulated by different components of the wnt pathway, as demonstrated in this study.
(3) The authors leveraged lineage tracing by photoconversion to dissect how dact1/2 differentially impacts the ability of different cranial neural crest populations to contribute to the anterior neurocranium. This revealed that distinct mechanisms via dact1/2 and shh can

lead to similar phenotypes.
(4) The use of scRNA-seq was a powerful approach and identified potential novel pathways and targets downstream of dact1/2.

Weaknesses:

(1) Expression of dact1/2 and wnt11f2: Certain claims regarding the expression similarity between dact2 and wnt11f2 is not clearly demonstrated in figures and the text description of dact1/2 and wnt11f2 expression for the Daniocell scRNA-seq tool is also somewhat confusing. As the paper makes claim that dact1/2 may function in the same pathway as wnt11f2, their expression should be accurately described and used to draw conclusion on what tissue types such a signaling may take place.
(2) Spatiotemporal function of dact1/2: Germline mutations limit the authors' ability to study a gene's spatiotemporal functional requirement. They, therefore, cannot concretely attribute nor separate early-stage phenotypes (during gastrulation) to/from late stage phenotypes (EP morphological changes), which the authors postulated to result from secondary defects in floor plate and eye field morphometry.
(3) The functional significance of calpain 8: The authors showed that calpain 8 was upregulated in the mutant and subsequently tested its function by overexpressing dact1/2 mRNA in embryos. While only 1 out of 142 calpain-overexpressing wild type animals phenocopied dact1/2 mutants, 7.5% of dact1/2+/- embryos did exhibit the phenotype. However, the same effect was not observed in dact1-/-; dact2+/- embryos and the requirement of calpain 8 in driving the phenotype remains unclear.

https://doi.org/10.7554/eLife.91648.2.sa2

Reviewer #3 (Public Review):

Summary:

In this manuscript the authors explore the roles of dact1 and dact2 during zebrafish gastrulation and craniofacial development. Previous studies used morpholino (MO) knockdowns to show that these scaffolding proteins, which interact with dishevelled (Dsh), are expressed during zebrafish gastrulation and suggested that dact1 promotes canonical Wnt/B-catenin signaling, while dact2 promotes non-canonical Wnt/PCP-dependent convergent-extension (Waxman et al 2004). This study goes beyond this work by creating loss-of-function mutant alleles for each gene and unlike the MO studies finds little (dact2) to no (dact1) phenotypic defects in the homozygous mutants. Interestingly, dact1/2 double mutants have a more severe phenotype, which resembles those reported with MOs as well as homozygous wnt11/silberblick (wnt11/slb) mutants that disrupt non-canonical Wnt signaling (Heisenberg et al., 1997; 2000). Further analyses in this paper try to connect gastrulation and craniofacial defects in dact1/2 mutants with wnt11/slb and other wnt-pathway mutants. scRNAseq conducted in mutants identifies calpain 8 as a potential new target of dact1/2 and Wnt signaling.

Previous comments:

Strengths:

When considered separately the new mutants are an improvement over the MOs and the paper contains a lot of new data.

Weaknesses:

However, the hypotheses are very poorly defined and misinterpret key previous findings surrounding the roles of wnt11 and gpc4, which results in a very confusing manuscript. Many

of the results are not novel and focus on secondary defects. The most novel result overexpressing calpain8 in dact1/2 mutants is preliminary and not convincing.

Comment on the revised version:

The authors addressed some of our comments, but not our main criticisms, which we reiterate here:

(1) The authors argue that morpholino studies are unreliable and here they made new mutants to solve this uncertainty for dap 1/2. However, creating stable mutant lines to largely confirm previous results obtained by using morpholino knock-down phenotypes does not justify publication in eLife.

(2) The authors argue that since it has not been shown conclusively that craniofacial defects in wnt11 and dap1/2 mutants are secondary to gastrulation defects there is no solid evidence preventing them from investigating these craniofacial defects. However, since it is extremely likely that the rod-like ethmoid plates of wnt11f2- and dact1/2 mutants focused on here are secondary to gastrulation defects previously described by others (Heisenberg and NussleinVolhard 1997; Waxman et al., 2004), the burden of proof is on the authors to provide much stronger evidence against this interpretation.

(3) The data for calpain overexpression remains too preliminary.

https://doi.org/10.7554/eLife.91648.2.sa1

Author response:

The following is the authors' response to the original reviews.

> ***Reviewer #1 (Public Review):***
>
> *Weakness 1. Enhancing Reproducibility and Robustness: To enhance the reproducibility and robustness of the findings, it would be valuable for the authors to provide specific numbers of animals used in each experiment. Explicitly stating the penetrance of the rod-like neurocranial shape in dact1/2-/- animals would provide a clearer understanding of the consistency of this phenotype.*

In Fig. 3 and Fig. 4 animal numbers were added to the figure and figure legend (line 1111). In Fig. 5 animal numbers were added to the figure. We now state that dact1/2-/- animals exhibit the rod-like neurocranial shape that is completely penetrant (Line 260).

> *Weakness 2. Strengthening Single-Cell Data Interpretation: To further validate the single-cell data and strengthen the interpretation of the gene expression patterns, I recommend the following:*
>
> *-Provide a more thorough explanation of the rationale for comparing dact1/2 double mutants with gpc4 mutants.*
>
> *-Employ genotyping techniques after embryo collection to ensure the accuracy of animal selection based on phenotype and address the potential for contamination of wild-type "delayed" animals.*
>
> *-Supplement the single-cell data with secondary validation using RNA in situ or immunohistochemistry techniques.*

An explanation of our rationale was added to the results section (Lines 391403) and a summary schematic was added to Figure 6 (panel A).

Genotyping of the embryos was not possible but quality control analysis by considering the top 2000 most variable genes across the dataset showed good clustering by genotype, indicating the reproducibility of individuals in each group (See Supplemental Fig. 4).

The gene expression profiles obtained in our single-cell data analysis for gpc4, dact1, and dact2 correlate closely with our in situ hybridization analyses. Further, our data is consistent with published zebrafish single-cell data. We validated our finding of increased capn8 expression in dact1/2 mutants by in situ hybridization. Therefore we are confident in the robustness of our single-cell data.

> *Weakness 3. Directly Investigating Non-Cell-Autonomous Effects: To directly assess the proposed non-cell-autonomous role of dact1/2, I suggest conducting transplantation experiments to examine the ability of ectodermal/neural crest cells from dact1/2 double mutants to form wild-type-like neurocranium.*

The reviewer's suggestion is an excellent experiment and something to consider for future work. Cell transplant experiments between animals of specific genotypes are challenging and require large numbers. It is not possible to determine the genotype of the donor and recipient embryos at the early timepoint of 1,000 cell stage where the transplants would have to be done in the zebrafish. So that each transplant will have to be carried out blind to genotype from a dact1+/-; dact2+/- or dact1-/-; dact2+/- intercross and then both animals have to be genotyped at a subsequent time point, and the phenotype of the transplant recipient be analyzed. While possible, this is a monumental undertaking and beyond the scope of the current study.

> *Weakness 4. Further Elucidating Calpain 8's Role: To strengthen the evidence supporting the critical role of Calpain 8, I recommend conducting overexpression experiments using a sensitized background to enhance the statistical significance of the findings.*

We thank the reviewer for their suggestion and have now performed capn8 overexpression experiments in embryos generated from dact1/2 double heterozygous breeding. We found a statistically significant effect of capn8 overexpression in the dact1+/-,dact2+/- fish (Lines 462-464 and Fig. 8C,D).

> *Minor Comments:*
>
> *Comment: Creating the manuscript without numbered pages, lines, or figures makes orientation and referencing harder.*

Revised

> *Comment: Authors are inconsistent in the use of font and adverbs, which requires extra effort from the reader. ("wntIIf2 vs wnt11f2 vs wnt11f2l"; "dact1/2-/- vs dact1/dact2 -/-"; "whole-mount vs wholemount vs whole mount").*

Revised throughout.

> *Comment: Multiple sentences in the "Results" belong to the "Materials and Methods" or the "Discussion" section.*

We have worked to ensure that sentences are within the appropriate sections of the manuscript.

> *Comment: Abstract:*
>
> *"wnt11f2l" should be "wnt11f2"*

Revised (Line 24).

> *Comment: Main text:*
>
> *Page 5 - citation Waxman, Hocking et al. 2004 is used 3x without interruption any other citation.*

Revised (Line 112).

> *Page 9 - "dsh" mutant is mentioned once in the whole manuscript - is this a mistake?*

Revised, Rewritten (Line 196).

> *Page 10 - Fig 2B does not show ISH.*

Revised (Line 229).

> *Page 11 - "kyn" mutant is mentioned here for the first time but defined on page 15.*

Revised (Line 245). Now first described on page 4.

> *Page 14 - "cranial CNN" should be CNCC.*

Revised. (Line 334)

> *Page 16 - dact1/dact2/gpc4: Fig. 5C is used but it should be Fig 5E.*

Revised. (Line 381)

> *Page 18 - dact1/2-/- or dact1-/-, dact2-/-.*

Revised. (Line 428)

> *Comment: Methods:*
>
> *Page 24 - ZIRC () "dot" is missing. ChopChop ")" is missing. "located near the 5' end of the gene" - In the Supplementary Figure 1 looks like in the middle of the gene.*

Revised. (Lines 600, 609, 611, respectively).

> *Page 25 - WISH -not used in the main text.*

Revised. (Line 346).

> *Page 26 - 4% (v/v) formaldehyde; at 4C - 4{degree sign}C; 50% (v/v) ethanol; 3% (w/v) methylcellulose.*

Revised. (Lines 659, 660, 662).

> *Page 27 - 0.1% (w/v) BSA.*

Revised. (Line 668).

> *Comment: Discussion:*
>
> *The overall discussion requires more references and additional hypotheses. On page 20, when mentioning 'as single mutants develop normally,' does this refer to the entire animals or solely the craniofacial domain? Are these mutants viable? If they are, it's crucial to discuss this phenomenon in relation to prior morpholino studies and genetic compensation.*
>
> *Observing how the authors interpret previously documented changes in nodal and shh signaling would be beneficial. While Smad1 is discussed, what about other downstream genes? Is shh signaling altered in the dact1/2 double mutants?*

We have revised the Discussion to include more references (Lines 473, 476, 483, 488, 491, 499, 501, 502, 510, 515, 529, 557, 558) and additional hypotheses (Lines 503-505, 511-519, 522-525). We have added more specific information regarding the single mutants (Lines 270-275, 480-493, Fig. S3). We have added discussion of other downstream genes, including smad1 (Lines 561-572) and shh (Lines 572-580).

> *Comment: Figures:*
>
> *Appreciating differences between specimens when eyes were or were not removed is quite hard.*

Yes this was an unfortunate oversight, however, the key phenotype is the EP shown in the dissections.

> *Fig 1. - wntIIf2 vs wnt11f2? C - Thisse 2001 - correct is Thisse et al. 2001.*

Revised typo in Fig 1. (And Line 1083).

> *Fig 1E: These plots are hard to understand without previous and detailed knowledge. Authors should include at least some demarcations for the cephalic mesoderm, neural ectoderm, mesenchyme, and muscle. Missing color code.*

We have moved this data to supplementary figure S1 and have added labels of the relevant cell types and have added the color code.

> *Comment:- Fig 2 - In the legend for C - "wildtype and dact2-/- mutant" and "dact1/2 mutant"; in the picture is dact1-/-, dact2-/-.*

Revised (Line 1105).

> *Fig 2 - B - it is a mistake in 6th condition dact1: 2x +/+, heterozygote (+/-) is missing.*

Revised Figure 2B.

> *Fig 4. - Typo in the legend: dact1/"t"2-/- .*

Revised. (Line 1127).

> *Fig 8C - In my view, when the condition gfp mRNA says "0/197, " none of the animals show this phenotype. I assume the authors wanted to say that all the animals show this phenotype; therefore, "197/197" should be used.*

We have removed this data from the figure as there were concerns by the reviewers regarding reproducibility.

> *Fig S1 - Missing legend for the 28 + 250, 380 + 387 peaks? RT-qPCR - is not mentioned in the Materials and Methods. In D - ratio of 25% (legend), but 35% (graph).*

Revised.(Line 1203, Line 625, Line 1213, respectively).

> *Fig S2 - The word "identified" - 2x in one sentence.*

Revised. (Line 1230).

> ***Reviewer #2 (Public Review):***
>
> *Weakness(1) While the qualitative data show altered morphologies in each mutant, quantifications of these phenotypes are lacking in several instances, making it difficult to gauge reproducibility and penetrance, as well as to assess the novel ANC forms described in certain mutants.*

In Fig. 3 and Fig. 4 animal numbers were added to the figure legend. In Fig. 5 animal numbers were added to the figure to demonstrate reproducibility. We now state that dact1/2-/- animals exhibit the rod-like neurocranial shape that is completely penetrant (Line 260). As the altered morphologies that we report are qualitatively significant from wildtype we did not find it necessary to make quantitative measurements. For experiments in which it was necessary to in-cross triple heterozygotes (Fig 3, Fig. 5), we dissected and visually analyzed the ANC of at least 3 compound mutant individuals. At least one individual was dissected for the previously published or described genotypes/phenotypes (i.e. wt, wntllf2-/-, dact1/2-/-, gpc4-/-, wls/-). We realize quantitative measurements may identify subtle differences between genotypes. However, the sheer number of embryos needed to generate these relatively rare combinatorial genotypes and the amount of genotyping required prevented quantitative analyses.

> *Weakness 2) Germline mutations limit the authors' ability to study a gene's spatiotemporal functional requirement. They therefore cannot concretely attribute nor separate early-stage phenotypes (during gastrulation) to/from late-stage phenotypes (ANC morphological changes).*

We agree that we cannot concretely attribute nor separate early and latestage phenotypes. Conditional mutants to provide temporal or cell-specific analysis are beyond the scope of this work. Here we speculate based on evidence obtained by comparing and contrasting embryos with grossly similar early phenotypes and divergent late-stage phenotypes. We believe our findings contribute to the existing body of literature on zebrafish mutants with both early convergent extension defects and craniofacial abnormalities.

> *Weakness (3) Given that dact1/2 can regulate both canonical and non-canonical wnt signaling, this study did not specifically test which of these pathways is altered in the dact1/2 mutants, and it is currently unclear whether disrupted canonical wnt signaling*

> *contributes to the craniofacial phenotypes, even though these phenotypes are typical non-canonical wnt phenotypes.*

Previous literature has attributed canonical wnt, non-canonical wnt, and nonwnt functions to dact, and each of these likely contributes to the dact mutant phenotype (Lines 87-89). We performed cursory analyses of tcf/lef:gfp expression in the dact mutants and did not find evidence to support further analysis of canonical wnt signaling in these fish. Single-cell RNAseq did not identify differential expression of any canonical or non-canonical wnt genes in the dact1/2 mutants.

Further research is needed to parse out the intracellular roles of dact1 and dact2 in response to wnt and tgf-beta signaling. Here we find that dact may also have a role in calcium signaling, and further experiments are needed to elaborate this role.

> *Weakness (4) The use of single-cell RNA sequencing unveiled genes and processes that are uniquely altered in the dact1/2 mutants, but not in the gpc4 mutants during gastrulation. However, how these changes lead to the manifested ANC phenotype later during craniofacial development remains unclear. The authors showed that calpain 8 is significantly upregulated in the mutant, but the fact that only 1 out of 142 calpainoverexpressing animals phenocopied dact1/2 mutants indicates the complexity of the system.*

To further test whether capn8 overexpression may contribute to the ANC phenotype we performed overexpression experiments in the resultant embryos of dact1/dact2 double het incross. We found the addition of capn8 caused a small but statistically significant occurrence of the mutant phenotype in dact1/2 double heterozygotes (Fig.8D). We agree with the reviewer that our results indicate a complex system of dysregulation that leads to the mutant phenotype. We hypothesize that a combination of gene dysregulation may be required to recapitulate the mutant ANC phenotype. Further, as capn8 activity is regulated by calcium levels, overexpression of the mRNA alone likely has a small effect on the manifestation of the phenotype.

> *Weakness (5) Craniofacial phenotypes observed in this study are attributed to convergent extension defects but convergent extension cell movement itself was not directly examined, leaving open if changes in other cellular processes, such as cell differentiation, proliferation, or oriented division, could cause distinct phenotypes between different mutants.*

Although convergent extension cell movements were not directly examined, our phenotypic analyses of the dact1/2 mutant are consistent with previous literature where axis extension anomalies were attributed to defects in convergent extension (Waxman 2004, Xing 2018, Topczewski 2001). We do not attribute the axis defect to differentiation differences as in situ analyses of established cell type markers show the existence of these cells, only displaced relative to wildtype (Figure 1). We agree that we cannot rule out a role for differences in apoptosis or proliferation however, we did not detect transcriptional differences in dact1/2 mutants that would indicate this in the single-cell RNAseq dataset. Defects in directed division are possible, but alone would not explain that dact1/2 mutant phenotype, particularly the widened dorsal axis (Figure 1).

> *Major comments:*
>
> *Comment (1) The author examined and showed convergent extension phenotype (CE) during body axis elongation in dact1/dact2-/- homozygous mutants. Given that dact2-/- single mutants also displayed shortened axis, the authors should either explain why they didn't analyze CE in dact2-/- (perhaps because that has been looked at in previously*

> *published dact2 morphants?) or additionally show whether CE phenotypes are present in dact1 and dact2 single mutants.*
>
> *The authors should quantify the CE phenotype in both dact2-/- single mutants and dact1/dact2-/- double mutants, and examine whether the CE phenotypes are exacerbated in the double mutants, which may lend support to the authors' idea that dact1 can contribute to CE. The authors stated in the discussion that they "posit that dact1 expression in the mesoderm is required for dorsal CE during gastrulation through its role in noncanonical Wnt/PCP signaling". However, no evidence was presented in the paper to show that dact1 influences CE during body axis elongation.*

Because any axis shortening in shortening in dact2-/- single mutants was overcome during the course of development and at 5 dpf there was no noticeable phenotype, we did not analyze the single mutants further.

We have added data to demonstrate the resulting phenotype of each combinatorial genotype to provide a more clear and detailed description of the single and compound mutants (Fig. S3).

Our hypothesis that dact1 may contribute to convergent extension is based on its apparent ability to compensate (either directly or indirectly) for dact2 loss in the dact2-/- single mutant.

> *Comment (2) Except in Fig. 2, I could not find n numbers given in other experiments. It is therefore unclear if these mutant phenotypes were fully or partially penetrant. In general, there is also a lack of quantifications to help support the qualitative results. For example, in Fig. 4, n numbers should be given and cell movements and/or contributions to the ANC should be quantified to statistically demonstrate that the second stream of CNCC failed to contribute to the ANC.*
>
> *Similarly, while the fan-shaped and the rod-shaped ANCs are very distinct, the various rod-shaped ANCs need to be quantified (e.g. morphometry or measurements of morphological features) in order for the authors to claim that these are "novel ANC forms", such as in the dact1/2-/-, gpc4/dact1/2-/-, and wls/dact1/2-/- mutants (Fig. 5).*

We have added n numbers for each experiment and stated that the rod-like phenotype of the dact1/2-/- mutant was fully penetrant.

Regarding CNCC experiments, we repeated the analysis on 3 individual controls and mutants and did not find evidence that CNCC migration was directly affected in the dact1/2 mutant. Rather, differences in ANC development are likely secondary to defects in floor plate and eye field morphometry. Therefore we did not do any further analyses of the CNCCs.

Regarding figure 5, we have added n numbers. We dissected and analyzed a minimum of three triple mutants (dact1/2-/-,gpc4-/- and dact1/2-/-,wls-/-) and numerous dact1/s double mutants and found that the triple mutant ANC phenotype was consistent and recognizably different enough from the dact1/2-/-, or gpc4 or wls single mutant that morphometry measurements were not needed. Further, the triple mutant phenotype (narrow and shortened) appears to be a simple combination of dact1/2 (narrow) and gpc4/wls (shortened) phenotypes. As we did not find evidence of genetic epistasis, we did not analyze the novel ANC forms further.

> *Comment (3): The authors have attributed the ANC phenotypes in dact1/2-/- to CE defects and altered noncanonical wnt signaling. However, no evidence was presented to support either. The authors can perhaps utilize diI labelling, photoconversionmediated lineage tracing, or live imaging to study cell movement in the ANC and compare that with the cell movement change in the gpc4-/- , and gpc4/dact1/2-/- mutants in order to first establish*

> *that dact1/2 affect CE and then examine how dact1/2 mutations can modulate the CE phenotypes in gpc4-/- mutants.*
>
> *Concurrently, given that dact1 and dact2 can affect (perhaps differentially) both canonical and non-canonical wnt signaling, the authors are encouraged to also test whether canonical wnt signaling is affected in the ANC or surrounding tissues, or at minimum, discuss the potential role/contribution of canonical wnt signaling in this context.*

Given the substantial body of research on the role of noncanonical wnt signaling and planar cell polarity pathway on convergent extension during axis formation (reviewed by Yang and Mlodzik 2015, Roszko et al., 2009) and the resulting phenotypes of various zebrafish mutants (i.e. Xing 2018, Topczewski 2001), including previous research on dact1 and 2 morphants (Waxman 2004), we did not find it necessary to analyze CE cell movements directly.

Our finding that CNCC migration was not defective in the dact1/2 mutants and the knowledge that various zebrafish mutants with anterior patterning defects (slb, smo, cyc) have a similar craniofacial abnormality led us to conclude that the rod-like ANC in the dact1/2 mutant was secondary to an early patterning defect (abnormal eye field morphology). Therefore, testing dact1/2 and convergent extension or wnt signaling in the ANC itself was not an aim of this paper.

> *Comment (4) The authors also have not ruled out other possibilities that could cause the dact1/2-/- ANC phenotype. For example, increased cell death or reduced proliferation in the ANC may result in the phenotype, and changes in cell fate specification or differentiation in the second CNCC stream may also result in their inability to contribute to the ANC.*

We agree that we cannot rule out whether cell death or proliferation is different in the dact1/2 mutant ANC. However, because we do not find the second CNCC stream within the ANC, this is the most likely explanation for the abnormal ANC shape. Because the first stream of CNCC are able to populate the ANC and differentiate normally, it is most likely that the inability of the second stream to populate the ANC is due to steric hindrance imposed by the abnormal cranial/eye field morphology. These hypotheses would need to be tested, ideally with an inducible dact1/2 mutant, however, this is beyond the scope of this paper.

> *Comment (5) The last paragraph of the section "Genetic interaction of dact1/2 with Wnt regulators..." misuses terms and conflates phenotypes observed. For instance, the authors wrote "dact2 haploinsuffciency in the context of dact1-/-; gpc4-/- double mutant produced ANC in the opposite phenotypic spectrum of ANC morphology, appearing similar to the gpc4-/- mutant phenotype". However, if heterozygous dact2 is not modulating phenotypes in this genetic background, its function is not "haploinsuffcient". The authors then said, "These results show that dact1 and dact2 do not have redundant function during craniofacial morphogenesis, and that dact2 function is more indispensable than dact1". However this statement should be confined to the context of modulating gpc4 phenotypes, which is not clearly stated.*

Revised (Lines 380, 382).

> *Comment (6) For the scRNA-seq analysis, the authors should show the population distribution in the UMAP for the 3 genotypes, even if there are no obvious changes. The authors are encouraged, although not required, to perform pseudotime or RNA velocity analysis to determine if differentiation trajectories are changed in the NC populations, in light of what they found in Fig. 4. The authors can also check the expression of reporter*

> *genes downstream of certain pathways, e.g. axin2 in canonical wnt signaling, to query if these signaling activities are changed (also related to point #3 above).*

We have added population distribution data for the 3 genotypes to Supplemental Figure 4. Although RNA velocity analysis would be an interesting additional analysis, we would hypothesize that the NC population is not driving the differences in phenotype. Rather these are likely changes in the anterior neural plate and mesoderm.

> *Comment (7) While the phenotypic difference between gpc4-/- and dact1/2-/- are in the ANC at a later stage, ssRNA-seq was performed using younger embryos. The authors should better explain the rationale and discuss how transcriptomic differences in these younger embryos can explain later phenotypes. Importantly, dact1, dact2, and capn8 expression were not shown in and around the ANC during its development and this information is crucial for interpreting some of the results shown in this paper. For example, if dact1 and dact2 are expressed during ANC development, they may have specific functions during that stage. Alternatively, if dact1 and dact2 are not expressed when the second stream CNCCs are found to be outside the ANC, then the ANC phenotype may be due to dact1/2's functions at an earlier time point. The author's statement in the discussion that "embryonic fields determined during gastrulation effect the CNCC ability to contribute to the craniofacial skeleton" is currently speculative.*

We have reworded our rationale and hypothesis to increase clarity (Lines 391-405). We believe that the ANC phenotype of the dact1/2 mutants is secondary to defective CE and anterior axis lengthening, as has been reported for the slb mutant (Heisenberg 1997, 2000). We utilized the gpc4 mutant as a foil to the dact1/2 mutant, as the gpc4 mutant has defective CE and axis extension without the same craniofacial phenotype.

We have added dact1 and dact2 WISH of 24 and 48 hpf (Fig1. D,E) to show expression during ANC development.

> *Comment (8) The functional testing of capn8 did not yield a result that would suggest a strong effect, as only 1 in 142 animals phenocopied dact1/2. Therefore, while the result is interesting, the authors should tone down its importance. Alternatively, the authors can try knocking down capn8 in the dact1/2 mutants to test how that affects the CE phenotype during axis elongation, as well as ANC morphogenesis.*

As overexpression of capn8 in wildtype animals did not result in a significant phenotype, we tested capn8 overexpression in compound dact1/2 mutants as these have a sensitized background. We found a small but statistically significant effect of exogenous capn8 in dact1+/-,dact2+/- animals. While the effect is not what one would expect comparing to Mendelian genetic ratios, the rod-like ANC phenotype is an extreme craniofacial dysmorphology not observed in wildtype or mRNA injected embryos hence significant. The experiment is limited by the available technology of over-expressing mRNA broadly without temporal or cell specificity control. It is possible that if capn8 over-expression was restricted to specific cells (floor plate, notochord or mesoderm) and at the optimal time period during gastrulation/segmentation that the aberrant ANC phenotype would be more robust. We agree with the reviewer that although the finding of a new role for capn8 during development is interesting, its importance in the context of dact should be toned down and we have altered the manuscript accordingly (Lines 455-467).

> *Comment (9) A difference between the two images in Fig. 8B is hard to distinguish.*
>
> *Consider showing flat-mount images.*

We have added flat-mount images to Fig. 8B

> *Minor comments:*
>
> *Comment (1) wnt11f2 is spelled incorrectly in a couple of places, e.g. "wnt11f2l" in the abstract and "wntllf2" in the discussion.*

Revised throughout.

> *Comment (2) For Fig. 1D, the white dact1 and yellow dact2 are hard to distinguish in the merged image. Consider changing one of their colors to a different one and only merge dact1 and dact2 without irf6 to better show their complementarity.*

We agree with the reviewer that the expression patterns of dact1 and dact2 are difficult to distinguish in the merged image. We have added outlines of the cartilage elements to the images to facilitate comparisons of dact1 and dact2 expression (Fig 1F).

> *Comment (3) For Fig. 1E, please label the clusters mentioned in the text so readers can better compare expressions in these cell populations.*

We have moved this data to supplementary figure S1 and have added labels.

> *Comment (4) The citing and labelling of certain figures can be more specific. For example, Fig. S1A, B, and Fig. S1C should be used instead of just Fig. S1 (under the section titled dact1 and dact2 contribute to axis extension…". Similarly, Fig. 4 can be better labeled with alphabets and cited at the relevant places in the text.*

We have modified the labeling of the figures according to the reviewer's suggestion (Fig S2 (previously S1), Fig4) and have added reference to these labels in the text (Lines 202, 204, 212, 328, 334, 336).

> *Comment (5) For Fig. 2B, the (+/+,-/-) on x-axis should be (+/-,-/-).*

Revised in Figure 2B.

> *Comment (6) Several figures are incorrectly cited. Fig. 2C is not cited, and the "Fig. 2C" and "Fig. 2D" cited in the text should be "Fig. 2D" and "Fig. 2E" respectively. Similarly, Fig. 5C and D are not cited in the text and the cited Fig. 5C should be 5E. The VC images in Fig. 5 are not talked about in the text. Finally, Fig. 7C was also not mentioned in the text.*

We have corrected the labeling and have added descriptions of each panel in the Results (Fig.2 Line 231, 237, 242, Fig 5 Line 373, 381, Fig 7 line 431).

> *Comment (7) In the main text, it is indicated that zebrafish at 3ss were used for ssRNAseq, but in the figure legend, it says 4ss.*

Revised (Line 682)

> *Comment (8) No error bars in Fig. S1B and the difference between the black and grey shades in Fig. S1D is not explained.*

Error bars are not included in the graphs of qPCR results (now Fig S2C) as these are results of a pool of 8 embryos performed one time. We have added a legend to explain the gray vs. black bars (now Fig S2E).

> ***Reviewer #3 (Public Review):***
>
> *Weaknesses: The hypotheses are very poorly defined and misinterpret key previous findings surrounding the roles of wnt11 and gpc4, which results in a very confusing manuscript. Many of the results are not novel and focus on secondary defects. The most novel result of overexpressing calpain8 in dact1/2 mutants is preliminary and not convincing.*

We apologize for not presenting the question more clearly. The Introduction was revised with particular attention to distinguish this work using genetic germline mutants from prior morpholino studies. Please refer to pages 4-5, lines 106-121.

> *Weakness 1) One major problem throughout the paper is that the authors misrepresent the fact that wnt11f2 and gpc4 act in different cell populations at different times. Gastrulation defects in these mutants are not similar: wnt11 is required for anterior mesoderm CE during gastrulation but not during subsequent craniofacial development while gpc4 is required for posterior mesoderm CE and later craniofacial cartilage morphogenesis (LeClair et al., 2009). Overall, the non-overlapping functions of wnt11 and gpc4, both temporally and spatially, suggest that they are not part of the same pathway.*

We have reworded the text to add clarity. While the loss of wnt11 versus the loss of gpc4 may affect different cell populations, the overall effect is a shortened body axis. We stressed that it is this similar impaired axis elongation phenotype but discrepant ANC morphology phenotypes in the opposite ends of the ANC morphologic spectrum that is very interesting and leads us to investigate dact1/2 in the genetic contexts of wnt11f2 and gpc4. Pls refer to page 4, lines 73-84. Further, the reviewer's comment that wnt11 and gpc4 are spatially and temporally distinct is untested. We think the reviewer's claim of gpc4 acting in the posterior mesoderm refers to its requirement in the tailbud (Marlow 2004). However this does not exclude gpc4 from acting elsewhere as well. Further experiments would be necessary. Both wnt11f2 and gpc4 regulate non-canonical wnt signaling and are coexpressed during some points of gastrulation and CF development (Gupta et al., 2013; Sisson 2015). This data supports the possibility of overlapping roles.

> *Weakness 2) There are also serious problems surrounding attempts to relate single-cell data with the other data in the manuscript and many claims that lack validation. For example, in Fig 1 it is entirely unclear how the Daniocell scRNA-seq data have been used to compare dact1/2 with wnt11f2 or gpc4. With no labeling in panel 1E of this figure these comparisons are impossible to follow. Similarly, the comparisons between dact1/2 and gpc4 in scRNA-seq data in Fig. 6 as well as the choices of DEGs in dact1/2 or gpc4 mutants in Fig. 7 seem arbitrary and do not make a convincing case for any specific developmental hypothesis. Are dact1 and gpc4 or dact2 and wnt11 coexpressed in individual cells? Eyeballing similarity is not acceptable.*

We have moved the previously published Daniocell data to Figure S1 and have added labeling. These data are meant to complement and support the WISH results and demonstrate the utility of using available public Daniocell data. Please recommend how we can do this better or recommend how we can remediate this work with specific comment.

Regarding our own scRNA-seq data, we have added rationale (line 391-403) and details of the results to increase clarity (Lines 419-436). We have added a panel to Figure 6 (panel A) to help illustrate or rationale for comparing dact1/2 to gpc4 mutants to wt. The DEGs displayed in Fig.7A are the top 50 most differentially expressed genes between dact1/2 mutants and WT (Figure 7 legend, line 422-424).

We have looked at our scRNA-seq gene expression results for our clusters of interest (lateral plate mesoderm, paraxial mesoderm, and ectoderm). We find dact1, dact2, and gpc4 co-expression within these clusters. Knowing whether these genes are coexpressed within the same individual cell would require going back and analyzing the raw expression data. We do not find this to be necessary to support our conclusions. The expression pattern of wnt11f2 is irrelevant here.

> *Weakness 3) Many of the results in the paper are not novel and either confirm previous findings, particularly Waxman et al (2004), or even contradict them without good evidence. The authors should make sure that dact2 loss-of-function is not compensated for by an increase in dact1 transcription or vice versa. Testing genetic interactions, including investigating the expression of wnt11f2 in dact1/2 mutants, dact1/2 expression in wnt11f2 mutants, or the ability of dact1/2 to rescue wnt11f2 loss of function would give this work a more novel, mechanistic angle.*

We clarified here that the prior work carried out by Waxman using morppholinos, while acceptable at the time in 2004, does not meet the rigor of developmental studies today which is to generate germline mutants. The reviewer's acceptance of the prior work at face value fails to take the limitation of prior work into account. Further, the prior paper from Waxman et al did not analyze craniofacial morphology other than eyeballing the shape of the head and eyes. Please compare the Waxman paper and this work figure for figure and the additional detail of this study should be clear. Again, this is by no means any criticism of prior work as the prior study suffered from the technological limitations of 2004, just as this study also is the best we can do using the tools we have today. Any discrepancies in results are likely due to differences in morpholino versus genetic disruption and most reviewers would favor the phenotype analysis from the germline genetic context. We have addressed these concerns as objectively as we can in the text (Lines 482-493). The fact that dact1/2 double mutants display a craniofacial phenotype while the single mutants do not, suggests compensation (Lines 503-505), but not necessarily at the mRNA expression level (Fig. S2C).

This paper tests genetic interaction through phenotyping the wntll/dact1/dact2 mutant.

Our results support the previous literature that dact1/2 act downstream of wnt11 signaling. There is no evidence of cross-regulation of gene expression. We do not expect that changes in wnt11 or dact would result in expression changes in the others.

RNA-seq of the dact1/2 mutants did not show changes in wnt11 gene expression. Unless dact1 and/or dact2 mRNA are under expressed in the wnt11 mutant, we would not expect a rescue experiment to be informative. And as wnt11 is not a focus of this paper, we have not performed the experiment.

> *Weakness 4) The identification of calpain 8 overexpression in Dact1/2 mutants is interesting, but getting 1/142 phenotypes from mRNA injections does not meet reproducibility standards.*

As the occurrence of the mutant phenotype in wildtype animals with exogenous capn8 expression was below what would meet reproducibility standards, we performed an additional experiment where capn8 was overexpressed in embryos resulting from dact1/dact2 double heterozygotes incross (Fig. 8). We reasoned that an effect of capn8 overexpression may be more robust on a sensitized background. We found a statistically significant effect of capn8 in dact1/2 double heterozygotes, though the occurrence was still relatively rare (6/80). These data suggest dysregulation of capn8 contributes to the mutant ANC phenotype, though there are likely other factors involved.

> *Comment: The manuscript title is not representative of the findings of this study.*

We revised the title to strictly describe that we generated and carried out genetic analysis in loss of function compound mutants (Genetic requirement) and that we found capn8 was important which modified this requirement.

> *Introduction: p.4:*
>
> *Comment: Anterior neurocranium (ANC) - it has to be stated that this refers to the combined ethmoid plate and trabecular cartilages.*

Thank you, we agree that the ANC and ethmoid plate terminology has been confusing in the literature and we should endeavor to more clearly describe that the phenotypes in question are all in the ethmoid plate and the trabeculae are not affected. ANC has been replaced with ethmoid plate (EP) throughout the manuscript and figures. We also describe that all the observed phenotypes affect the ethmoid plate and not the trabeculae, (pages 13, Lines 265-267).

> *Comment: Transverse dimension is incorrect terminology - replace with medio-lateral.*

Revised (Lines 69, 74).

> *Comment: Improper way of explaining the relationship between mutant and gene..."Another mutant knypek, later identified as gpc4..." a better way to explain this would be that the knypek mutation was found to be a non-sense mutation in the gpc4 gene.*

Revised (Line 71)

> *Comment: "...the gpc4 mutant formed an ANC that is wider in the transverse dimension than the wildtype, in the opposite end of the ANC phenotypic spectrum compared to wnt11f2...These observations beg the question how defects in early patterning and convergent extension of the embryo may be associated with later craniofacial morphogenesis."*
>
> *This statement is broadly representative of the general failure to distinguish primary from secondary defects in this manuscript. Focusing on secondary defects may be useful to understand the etiology of a human disease, but it is misleading to focus on secondary defects when studying gene function. The rod-like ethmoid of slb mutant results from a CE defect of anterior mesoderm during gastrulation(Heisenberg et al. 1997, 2000), while the wide ethmoid plate of kny mutants results from CE defects of cartilage precursors (Rochard et al., 2016). Based on this evidence, wnt11f2 and gpc4 act in different cell populations at different times.*

It is true that the slb mutant craniofacial phenotype has been stated as secondary to the CE defect during gastrulation and the kny phenotype as primary to chondrocyte CE defects in the ethmoid, however the direct experimental evidence to conclude only primary or only secondary effects does not yet exist. There is no experiment to our knowledge where wnt11f2 was found to not affect ethmoid chondrocytes directly. Likewise, there is no experiment having demonstrated that dysregulated CE in gpc4 mutants does not contribute to a secondary abnormality in the ethmoid.

Here, we are analyzing the CE and craniofacial phenotypes of the dact1/2 mutants without any assumptions about primary or secondary effects and without drawing any conclusions

about wnt11f2 or gpc4 cellular mechanisms.

> *Comment: "The observation that wnt11f2 and gpc4 mutants share similar gastrulation and axis extension phenotypes but contrasting ANC morphologies supports a hypothesis that convergent extension mechanisms regulated by these Wnt pathway genes are specific to the temporal and spatial context during embryogenesis."*
>
> *This sentence is quite vague and potentially misleading. The gastrulation defects of these 2 mutants are not similar - wnt11 is required for anterior mesoderm CE during gastrulation and has not been shown to be active during subsequent craniofacial development while gpc4 is required for posterior mesoderm CE and craniofacial cartilage morphogenesis (LeClair et al., 2009). Here again, the non-spatially overlapping functions of wnt11 and gpc4 suggest that are not part of the same pathway.*

Though the cells displaying defective CE in wnt11f2 and gpc4 mutants are different, the effects on the body axis are similar. The dact1/2 showed a similar axis extension defect (grossly) to these mutants. Our aim with the scRNA-seq experiment was to determine which cells and gene programs are disrupted in dact1/2 mutants. We found that some cell types and programs were disrupted similarly in dact1/2 mutants and gpc4 mutants, while other cells and programs were specific to dact1/2 versus gpc4 mutants. We can speculate that these that were specific to dact1/2 versus gpc4 may be attributed to CE in the anterior mesoderm, as is the case for wnt11.

> *p.5*
>
> *Comment: "We examined the connection between convergent extension governing gastrulation, body axis segmentation, and craniofacial morphogenesis." A statement focused on the mechanistic findings of this paper would be welcome here, instead of a claim for a "connection" that is vague and hard to find in the manuscript.*

We have rewritten this statement (Line 125).

> *p.7 Results:*
>
> *Comment: It is unclear why Farrel et al., 2018 and Lange et al., 2023 are appropriate references for WISH. Please justify or edit.*

This was a mistake and has been edited (Page 9).

> *Comment: " Further, dact gene expression was distinct from wnt11f2." This statement is inaccurate in light of the data shown in Fig1A and the following statements - please edit to reflect the partially overlapping expression patterns.*

We have edited to clarify (Lines 142-143).

> *p.8*
>
> *Comment: "...we examined dact1 and 2 expression in the developing orofacial tissues. We found that at 72hpf..." - expression at 72hpf is not relevant to craniofacial morphogenesis, which takes place between 48h-60hpf (Kimmel et al., 1998; Rochard et al., 2016; Le Pabic et al., 2014).*

We have included images and discussion of dact1 and dact2 expression at earlier time points that are important to craniofacial development (Lines 160-171)(Fig 1D,E).

> *Comment: "This is in line with our prior finding of decreased dact2 expression in irf6 null embryos". - This statement is too vague. How are th.e two observations "in line".*

We have removed this statement from the manuscript.

> *Comment: Incomplete sentence (no verb) - "The differences in expression pattern between dact1 and dact2...".*

Revised (Line 172).

> *Comment: "During embryogenesis..." - Please label the named structures in Fig.1E.*
>
> *Please be more precise with the described expression time. Also, it would be useful to integrate the scRNAseq data with the WISH data to create an overall picture instead of treating each dataset separately.*

We have moved the previously published Daniocell data to supplementary figure S1 and have labeled the key cell types.

> *p.9*
>
> *Comment: "The specificity of the gene disruption was demonstrated by phenotypic rescue with the injection of dact1 or dact2 mRNA (Fig. S1)." - please describe what is considered a phenotypic rescue.*
>
> *-The body axis reduction of dact mutants needs to be documented in a figure. Head pictures are not sufficient. Is the head alone affected, or both the head and trunk/tail? Fig.2E suggests that both head and trunk/tail are affected - please include a live embryos picture at a later stage.*

We have added a description of how phenotypic rescue was determined (Line 208). We have added a figure with representative images of the whole body of dact1/2 mutants. Measurements of body length found a shortening in dact1/2 double mutants versus wildtype, however differences were not found to be significantly different by ANOVA (Fig. 3C, Fig. S3, Line 270-275).

> *p. 11*
>
> *Comment: "These dact1-/-;dact2-/- CE phenotypes were similar to findings in other Wnt mutants, such as slb and kny (Heisenberg, Tada et al., 2000; Topczewski, Sepich et al., 2001)." The similarity between slb and kny phenotypes should be mentioned with caution as CE defects affect different regions in these 2 mutants. It is misleading to combine them into one phenotype category as wnt11 and gpc4 are most likely not acting in the same pathway based on these spatially distinct phenotypes.*

Here we are referring to the grossly similar axis extension defects in slb and kny mutants. We refer to these mutants to illustrate that dact1 and or 2 deficiency could affect axis extension through diverse mechanisms. We have added text for clarity (Lines 249-252).

> *Comment: "No craniofacial phenotype was observed in dact1 or dact2 single mutants. However, in-crossing to generate [...] compound homozygotes resulted in dramatic craniofacial deformity."*
>
> *This result is intriguing in light of (1) the similar craniofacial phenotype previously reported by Waxman et al (2004) using morpholino- based knock-down of dact2, and the*

> *phenomenon of genetic compensation demonstrated by Jakutis and Stainier 2001 (https://doi.org/10.1146/annurev-genet-071719-020342). The authors should make sure that dact2 loss-of-function is not compensated for by an increase in dact1 transcription, as such compensation could lead to inaccurate conclusions if ignored.*

We agree with the reviewer that genetic compensation of dact2 by dact1 likely explains the different result found in the dact2 morphant versus CRISPR mutant. We found increased dact1 mRNA expression in the dact2-/- mutant (Fig S2X) however a more thorough examination is required to draw a conclusion. Interestingly, we found that in wildtype embryos dact1 and dact2 expression patterns are distinct though with some overlap. It would be informative to investigate whether the dact1 expression pattern changes in dact2-/- mutants to account for dact2 loss.

> *Comment: "Lineage tracing of NCC movements in dact1/2 mutants reveals ANC composition" - the title is misleading - ANC composition was previously investigated by lineage tracing (Eberhardt et al., 2006; Wada et al., 2005).*

This has been reworded (Line 292)

> *p.13*
>
> *Comment: There is no frontonasal prominence in zebrafish.*

This is true, texts have been changed to frontal prominence. (Lines 293,

> *Comment: The rationale for investigating NC migration in mutants where there is a gastrula-stage failure of head mesoderm convergent extension is unclear. The whole head is deformed even before neural crest cells migrate as the eye field does not get split in two (Heisenberg et al., 1997; 2000), suggesting that the rod-like ethmoid plate is a secondary defect of this gastrula-stage defect. In addition, neural crest migration and cartilage morphogenesis are different processes, with clear temporal and spatial distinctions.*

We carried out the lineage tracing experiment to determine which NC streams contributed to the aberrantly shaped EP, whether the anteromost NC stream frontal prominence, the second NC stream of maxillary prominence, or both. We found that the anteromost NCC did contribute to the rod-like EP, which is different from when hedgehod signaling is disrupted, So while it is possible that the gastrula-effect head mesoderm CE caused a secondary effect on NC migration, how the anterior NC stream and second NC stream are affected differently between dact1/2 and shh pathway is interesting. We added discussion of this observation to the manuscript (page 23, Lines 514-520).

> *p. 14-16*
>
> *Comment: Based on the heavy suspicion that the rod-like ethmoid plate of the dact1/2 mutant results from a gastrulation defect, not a primary defect in later craniofacial morphogenesis, the prospect of crossing dact1/2 mutants with other wnt-pathway mutants for which craniofacial defects result from craniofacial morphogenetic defects is at the very least unlikely to generate any useful mechanistic information, and at most very likely to generate lots of confusion. Both predictions seem to take form here.*
>
> *However, the ethmoid plate phenotype observed in the gpc4-/-; dact1+/-; dact2-/- mutants (Fig. 5E) does suggest that gpc4 may interact with dact1/2 during gastrulation, but that is the case only if dact1+/-; dact2-/- mutants do not have an ethmoid cartilage defect, which I could not find in the manuscript. Please clarify.*

The perspective that the rod-like EP of the dact1/2 is due to gastrulation defect is being examined here. Why would other mutants such as wnt11f2 and gpc4 that have gastrulation CE defects have very different EP morphology, whether primary or secondary NCC effect? Further dact1 and dact2 were reported as modifiers of Wnt signaling, so it is logical to genetically test the relationship between dact1, dact2, wnt11f2, gpc4 and wls. The experiment had to be done to investigate how these genetic combinations impact EP morphology. This study found that combined loss of dact1, dact2 and wls or gpc4 yielded new EP morphology different than those previously observed in either dact1/2, wls, gpc4, or any other mutant is important, suggesting that there are distinct roles for each of these genes contributing to facial morphology, that is not explained by CE defect alone.

> *Comment: I encourage the authors to explore ways to test whether the rod-like ethmoid of dact1/2 mutants is more than a secondary effect of the CE failure of the head mesoderm during gastrulation. Without this evidence, the phenotypes of dact1/2 -gpc4 or - wls are not going to convince us that these factors actually interact.*

Actually, we find our results to support the hypothesis that the ethmoid of the dact1/2 mutants is a secondary effect of defective gastrulation and anterior extension of the body axis. However, our findings suggest (by contrasting to another mutant with impaired CE during gastrulation) that this CE defect alone cannot explain the dysmorphic ethmoid plate. Our single-cell RNA seq results and the discovery of dysregulated capn8 expression and proteolytic processes presents new wnt-regulated mechanisms for axis extension.

> *p. 20 Discussion*
>
> *Comment: "Here we show that dact1 and dact2 are required for axis extension during gastrulation and show a new example of CE defects during gastrulation associated with craniofacial defects."*
>
> *Waxman et al. (2004) previously showed that dact2 is involved in CE during gastrulation.*
>
> *Heisenberg et al. (1997, 2000), previously showed with the slb mutant how a CE defect during gastrulation causes a craniofacial defect.*

The Waxman paper using morpholino to disrupt dact2 is produced limited analysis of CE and no analysis of craniofacial morphogenesis. We generated genetic mutants here to validate the earlier morpholino results and to analyze the craniofacial phenotype in detail. We have removed the word “new” to make the statement more clear (Line 475).

> *Comment: "Our data supports the hypothesis that CE gastrulation defects are not causal to the craniofacial defect of medially displaced eyes and midfacial hypoplasia and that an additional morphological process is disrupted."*
>
> *It is unclear to me how the authors reached this conclusion. I find the view that medially displaced eyes and midfacial hypoplasia are secondary to the CE gastrulation defects unchallenged by the data presented.*

This statement was removed and the discussion was reworded.

> *Comment: The discussion should include a detailed comparison of this study's findings with those of zebrafish morpholino studies.*

We have added more discussion to compare ours to the previous morpholino findings (Lines 476-484).

> *Comment: The discussion should try to reconcile the different expression patterns of dact1 and dact2, and the functional redundancy suggested by the absence of phenotype of single mutants. Genetic compensation should be considered (and perhaps tested).*

The different expression patterns of dact1 and dact2 along with our finding that dact1 and dact2 genetic deficiency differently affect the gpc4 mutant phenotype suggest that dact1 and dact2 are not functionally redundant during normal development. This is in line with the previously published data showing different phenotypes of dact1 or dact2 knockdown. However, our results that genetic ablation of both dact1 and dact2 are required for a mutant phenotype suggests that these genes can compensate upon loss of the other. This would suggest then that the expression pattern of dact1 would be changed in the dact2 mutant and visa versa. We find that this line of investigation would be interesting in future studies. We have addressed this in the Discussion (Lines 485498).

> *Comment: "Based on the data...Conversely, we propose...ascribed to wnt11f2 "*
>
> *Functional data always prevail overexpression data for inferring functional requirements.*

This is true.

> *p.21*
>
> *Comment: "Our results underscore the crucial roles of dact1 and dact2 in embryonic development, specifically in the connection between CE during gastrulation and ultimate craniofacial development."*
>
> *How is this novel in light of previous studies, especially by Waxman et al. (2004) and Heisenberg et al. (1997, 2000). In this study, the authors fail to present compelling evidence that craniofacial defects are not secondary to the early gastrulation defects resulting from dact1/2 mutations. p. 22*

We have not claimed that the craniofacial defects are not secondary to the gastrulation defects. In fact, we state that there is a “connection”. Further, we do not claim that this is the first or only such finding. We believe our findings have validated the previous dact morpholino experiments and have contributed to the body of literature concerning wnt signaling during embryogenesis.

> *Comment: The section on Smad1 discusses a result not reported in the results section. Any data discussed in the discussion section needs to be reported first in the results section.*

We have added a comment on the differential expression of smad1 to the results section (Lines 446-448).

https://doi.org/10.7554/eLife.91648.2.sa0