RESEARCH ARTICLE

Variation in phenotypes from a Bmp-Gata3 genetic pathway is modulated by Shh signaling

Mary E. Swartz*, C. Ben Lovely¤, Johann K. Eberhart

Department of Molecular Biosciences, University of Texas at Austin, Austin, Texas, United States of America

¤ Current Address: Department of Biochemistry and Molecular Genetics, University of Louisville, Louisville, Kentucky, United States of America

* swartz@austin.utexas.edu

Abstract

We sought to understand how perturbation of signaling pathways and their targets generates variable phenotypes. In humans, GATA3 associates with highly variable defects, such as HDR syndrome, microsomia and choanal atresia. We previously characterized a zebrafish point mutation in gata3 with highly variable craniofacial defects to the posterior palate. This variability could be due to residual Gata3 function, however, we observe the same phenotypic variability in gata3 null mutants. Using hsp:GATA3-GFP transgenics, we demonstrate that Gata3 function is required between 24 and 30 hpf. At this time maxillary neural crest cells fated to generate the palate express gata3. Transplantation experiments show that neural crest cells require Gata3 function for palatal development. Via a candidate approach, we determined if Bmp signaling was upstream of gata3 and if this pathway explained the mutant’s phenotypic variation. Using BRE:d2EGFP transgenics, we demonstrate that maxillary neural crest cells are Bmp responsive by 24 hpf. We find that gata3 expression in maxillary neural crest requires Bmp signaling and that blocking Bmp signaling, in hsp:DN-Bmpr1a-GFP embryos, can phenocopy gata3 mutants. Palatal defects are rescued in hsp:DN-Bmpr1a-GFP;hsp:GATA3-GFP double transgenic embryos, collectively demonstrating that gata3 is downstream of Bmp signaling. However, Bmp attenuation does not alter phenotypic variability in gata3 loss-of-function embryos, implicating a different pathway. Due to phenotypes observed in hypomorphic shha mutants, the Sonic Hedgehog (Shh) pathway was a promising candidate for this pathway. Small molecule activators and inhibitors of the Shh pathway lessen and exacerbate, respectively, the phenotypic severity of gata3 mutants. Importantly, inhibition of Shh can cause gata3 haploinsufficiency, as observed in humans. We find that gata3 mutants in a less expressive genetic background have a compensatory upregulation of Shh signaling. These results demonstrate that the level of Shh signaling can modulate the phenotypes observed in gata3 mutants.
Author summary

Human birth defects vary widely in their presentation. This is true even in cases where the underlying genetic mutation is the same. In humans, mutation of the gene GATA3 associates with two highly variable birth defects that can disrupt development of the face, microsomia and Hypoparathyroidism, Deafness and Renal dysplasia (HDR) syndrome. We used the zebrafish to identify the causes of variation in facial defects associated with gata3. We show that the cells that generate the palate require the function of Gata3 and that the Bone Morphogenetic Protein (Bmp) pathway is necessary for the expression of gata3 by these cells. While Gata3 functions downstream of Bmp, we find no evidence that alteration of the Bmp pathway causes the variability in skeletal defects in gata3 mutants. Instead, we identify a separate signaling pathway, the Sonic Hedgehog (Shh), pathway that is responsible for the variability in gata3 mutant defects. In a genetic background that promotes mild gata3 mutant phenotypes, Shh signaling is elevated relative to mutants in a genetic background sensitized for severe defects. Reduction or elevation of Shh signaling in these two mutants, exacerbates and lessens the phenotypic severity, respectively. Thus, our finding provides important insight into how interactions between signaling pathways cause variation in human birth defects.

Introduction

Congenital birth defects are a leading cause of infant mortality worldwide and the leading cause of mortality in many industrial nations according to the World Health Organization. The causes of most birth defects are thought to be complex and include genetic and environmental risk factors. Furthermore, the precise phenotypes observed within a specific birth defect can be highly variable and this variability is also thought to arise from genetic and environmental modifiers. Craniofacial defects are among the most common birth defects and offer an excellent model of variability. For instance, orofacial clefts affect 1 in 700 live births and appear to be caused by an interplay of genetic and environmental factors [1].

The high rate and variable nature of craniofacial defects such as orofacial clefts are largely because proper palatogenesis requires the precise coordination of many events that are subject to genetic and/or environmental perturbations. Cranial neural crest cells (CNCC) that generate the palatal skeleton are generated in the dorsal neural tube from which they must migrate into the periphery to differentiate. Palatal precursors occupy the maxillary region of the first pharyngeal arch and the frontonasal prominence in human, mouse and zebrafish [1–4]. The zebrafish palate (also referred to as the anterior neurocranium) is comprised of an anterior, midline, ethmoid plate and the posterior bilateral trabeculae. Fate mapping shows that the medial ethmoid palate is formed from frontonasal CNCC and the remaining palate forms from maxillary CNCC [2,3]. The trabeculae fuse to the posterior neurocranium which is primarily composed of mesodermally-derived cells [5]. While the evolutionary homologies remain unclear, a growing body of evidence demonstrates that the gene function required for craniofacial development, including palatogenesis, in mammals is conserved in zebrafish [6–9]. Yet we still have an incomplete knowledge of the genes involved in craniofacial development and a poor understanding of how they interact to generate variability.

Defining the causes of phenotypic variation is important for our understanding of development, disease and evolution. However, there are a limited number of studies defining the cause of phenotypic variation. Such variation can conceptually be caused by three general
mechanisms: 1) genetic background, 2) gene-environment interactions and 3) stochastic developmental events [10]. Our understanding of all of these mechanisms is limited. Recent work is beginning to shed light on the mechanisms of gene-environment interactions and how such interactions can synergistically effect phenotypes [11,12]. Similarly, mutant analyses in mouse and zebrafish have pointed to the importance of genetic background, with phenotypes differing depending upon the strain carrying the mutation [13–16]. Recent studies have demonstrated that selective breeding for heritable variation in phenotypic penetrance of mef2ca mutants results in altered methylation in a transposon at the mef2ca promoter and a compensatory downregulation of the opposing Notch pathway [17,18], providing some insights into these mechanisms. However, much remains to be understood regarding the nature of these genetic background effects.

The zinc finger transcription factor, GATA3, associates with craniofacial syndromes. Haploinsufficiency of GATA3 causes Hypoparathyroidism, Deafness and Renal dysplasia (HDR) syndrome [19]. HDR is an extremely variable birth defect, even among individuals sharing the same mutation within a family [20]. Palatal defects and choanal atresia (defects of the nasal bones) are craniofacial defects that can co-occur with the HDR triad [21,22]. Furthermore, GATA3 associates with craniofacial microsomia [23–25], another highly variable disease. Humans with microsomia can have unilateral shortening and clefts of the palate as well as defects to other craniofacial bones, ears and cranial ganglia [24,26]. While HDR is a relatively rare disease microsomia is very common affecting 1 in 5600 conceptuses. Collectively, these findings in human patients suggest that phenotypes associated with loss of GATA3 function are inherently variable.

Our understanding of the roles of GATA3 in craniofacial development is limited due to the early lethality of mouse Gata3 mutants caused by parathyroid defects [27,28]. Gata3 mutant mice pharmacologically rescued display severe craniofacial defects and neural crest patterning defects [28,29], consistent with a critical role in craniofacial development. Work in the mouse mandible has demonstrated that Smad1/5 binds regions adjacent to Gata3, suggesting that it is a target of Bmp signaling [30] furthermore Gata3 is a BMP target in limb mesenchyme [31]. However, the precise roles of Gata3, its regulation during palate development and the modulation of resulting phenotypes remain unknown.

We previously demonstrated that phenotypes in zebrafish gata3 mutants were highly variable, similar to human disorders associated with GATA3, and that this variability associated with genetic background [13]. Here, we determine the role of gata3 in development of the zebrafish palate and characterize the signaling pathways that regulate the variability in gata3 mutant phenotypes. We show that neural crest cells require the function of Gata3 shortly after their migration into the pharyngeal arches. Bmp signaling is necessary for the expression of gata3 in the maxillary neural crest and loss of Bmp signaling recapitulates the craniofacial defects in gata3 mutants. Transgenic overexpression of GATA3 restores facial development in Bmp deficient zebrafish, demonstrating that Gata3 functions downstream of Bmp. We demonstrate that the variability in gata3 mutant phenotypes is due to the actions of a second pathway, Shh. Elevating and attenuating Shh signaling ameliorates and exacerbates the phenotypes of gata3 mutants, respectively. Importantly, in a sensitized gata3 mutant genetic background, reduction of Shh signaling is sufficient to cause gata3 haploinsufficiency, similar to the human condition. Our results demonstrate that the coordination of two pathways, a Bmp-Gata3 pathway and Shh, regulate trabeculae phenotypes. These findings provide important insights into the causes of variability in craniofacial disease phenotypes.
Results

Loss of Gata3 results in highly unstable palatal phenotypes

Previously, we reported a missense mutant allele of gata3, b1075, [13] (Fig 1A) that displayed highly variable palatal phenotypes. We demonstrated that selective breeding across genetic backgrounds, could be used to select for separate populations that consistently produce phenotypes at each end of this spectrum. These phenotypes ranged from a severe truncation of the trabeculae, resulting in the failure of trabeculae to fuse to the mesoderm-derived posterior neurocranium (which we refer to as a cleft for simplicity) in one population, to mild cell rearrangements within the trabeculae, in a second population [13]. The gata3\textsuperscript{b1075} allele disrupts a cysteine that coordinates the zinc ion in the DNA-binding zinc finger of Gata3. The nature of this mutation mirrored a human \textit{GATA3} mutation demonstrated to lack DNA binding capability in cell culture [32]. However, Gata transcription factors have been shown to effect gene expression without DNA binding, via protein-protein interactions [33]. Thus, it remained plausible that the variability in the zebrafish mutant was due to residual Gata3 function within an \textit{in vivo} context.

We generated a new allele of gata3 using CRISPR/Cas-9. We targeted the second exon of gata3 to delete both zinc finger domains and generated the allele, au42, that has a two base pair insertion followed by a 25 base pair deletion (Fig 1B). By sequencing mutant mRNA, we confirmed the indel and identified four in frame stop codons following the indel (Fig 1C). The predicted truncated protein lacks both zinc finger domains, which are essential for function. Thus, we conclude that au42 is a null allele of gata3.

Interestingly, gata3\textsuperscript{au42} mutants exhibited the full spectrum of phenotypes observed in gata3\textsuperscript{b1075} mutants (Fig 2; [13]. In/del mutations, such as au42, can be associated with nonsense-mediated decay, which can result in genetic compensation [34]. However, the b1075 allele is a mis-sense mutation, unlikely to undergo nonsense mediated decay. Thus, the similarity in phenotypes suggests a mechanism other than genetic compensation following nonsense mediated decay for the phenotypic variability.
As with gata3 b1075 mutants, gata3 au42 mutant craniofacial phenotypes appear restricted to the trabeculae of the zebrafish palate and the lateral commissure. In zebrafish with the least severe phenotype the trabeculae fuses appropriately to the posterior neurocranium but is shortened and the cells within the trabeculae are inappropriately stacked but are in the same plane as and fused to the posterior neurocranium with all cells in one plane. (C) Trabeculae cells not stacked properly and fused inappropriately, below the plane of the posterior neurocranium, see arrowhead (score = 1). (D) Loss of trabeculae cells (score = 0) and therefore no fusion to the posterior neurocranium. (E-G) 4 dpf flat mounted neurocrania, anterior to the left showing examples of trabeculae scoring. (E) Wild type zebrafish embryos, arrowhead in G indicating improper lateral commissure fusion. In subsequent figures the numbering above the trabeculae reflect trabeculae scoring.

As with gata3^{b1075} mutants, gata3^{au42} mutant craniofacial phenotypes appear restricted to the trabeculae of the zebrafish palate and the lateral commissure. In zebrafish with the least severe phenotype the trabeculae fuses appropriately to the posterior neurocranium but is shortened and the cells within the trabeculae are inappropriately stacked but are in the same plane as and fused to the posterior neurocranium (Fig 2B and 2F; for our quantification below this is scored as a 2). We observed an intermediate phenotype in which the trabeculae are intact but have inappropriately stacked cells that fuse incorrectly to the ventral side of the posterior neurocranium so that the trabeculae and the posterior neurocranium are not in the same plane. (Fig 2C, arrowhead, and 2G; scored as a 1). The most severe phenotype is the cleft of the trabeculae in which the trabeculae are shortened and do not fuse to the posterior neurocranium (Fig 2D, 2F and 2G; scored as a 0). Despite the variability in the trabeculae phenotypes, which we focus on here, we note that mutants are 100% penetrant for a defect in which the lateral commissure fuses inappropriately to a more anterior region of the neurocranium. Compare arrowhead in Fig 2E indicating the wild type fusion of lateral commissure cell to arrowhead in Fig 2G indicating improper fusion.

We further characterized the nature of the phenotypic variability by comparing phenotypes within larvae. We compared the score from the left and right trabeculae of 39 neurocrania (Table 1). Neurocrania with concordant scores (green boxes) are slightly less numerous (n = 18) than those neurocrania that have discordant scores (blue and yellow boxes, n = 21). Neurocrania scores differing by more than one (yellow boxes) are uncommon. This asymmetry fluctuates in its sidedness. However, the left side tends to have a less severe phenotype.

Neural crest cells require the function of Gata3

To begin to understand how Gata3 functions during palatal development we assayed its spatio-temporal expression during craniofacial development. Riboprobes against gata3 label the head by 22 hpf (Fig 3A). However, at this time the expression appears to be in neural progenitors (black arrow), the ear (e) and what is likely to be endoderm (asterisk). Neural crest expression
of gata3 begins by 24 hpf and continues until 48 hpf (Fig 3B–3D). At 24 hpf, neural crest cells just ventral to the eye begin expressing gata3 transcripts and this staining becomes prominent by 26 hpf (Fig 3B and 3C, white arrowheads). This expression domain matches the region fate mapped to become the anterior neurocranium, particularly the trabeculae, via single cell fate mapping at 24 hpf [2,3] and kaede photoconversion at 36 hpf [6]. By 48 hpf, when the progenitors of the trabeculae have extended into their rod-shaped structure [2,6], the expression of gata3 becomes restricted away from these precursors (Fig 3D). To verify that maxillary neural crest cells express gata3 we performed fluorescent in situ hybridization on 36 hpf embryos using probes to gata3 and pdgfra, as a marker of neural crest cells. The overlap of gata3 and pdgfra confirms that gata3 is expressed in maxillary crest cells. Thus, the expression pattern of gata3 in the early maxillary domain suggests a role in the cell behaviors that mediate extension of the zebrafish palatal skeleton.

Given the dynamic nature of the expression of gata3, we sought to determine when palatal development requires Gata3 function. We generated a hsp:GATA3-EGFP heat shock transgenic line to temporally regulate the expression of GATA3 (Fig 4). Three hours following heat shock, the GATA3-EGFP fusion protein clearly localized to cell nuclei (Fig 4A), consistent with it being a functional fusion protein. To determine when palate development requires GATA3 function, we determined when transgenic expression of GATA3-EGFP could rescue the phenotype of gata3au42 mutants. Embryos were heat shocked at times points ranging from 18 hpf, as a control for a time point prior to neural crest expression of gata3, to 48 hpf and then grown up to 4 dpf. All embryos were stained and trabeculae on each side of the palate were scored according to the system outlined in Fig 2. We found that ectopic expression of GATA3 had no apparent effect on the phenotypes of embryos wild type for gata3 (Fig 4B) and (S1 Fig). Heat shock prior to 24 hpf did not improve the development of the trabeculae (Fig 4D). The trabeculae phenotype was most significantly rescued by heat shock at 24 hpf (Fig 4C and 4D), although there was some rescue as late as 30 hpf. See Table 2 for a complete list of statistics. Thus, Gata3 appears to function over a small window of time when maxillary neural crest cells are condensing in the pharyngeal arches to promote trabeculae development.

Maxillary neural crest cells express gata3 during the period when it is required for trabeculae development. To determine if cranial neural crest cells require gata3 autonomously, we created genetic chimeras. We transplanted membrane labeled sox10:mCherry CNCC into fli1:EGFP;gata3 mutant hosts unilaterally (Fig 5). This allowed us to compare the phenotype on the control side, comprised of all mutant cells, to the transplanted side, chimeric for mutant and wild type cells. Embryos were imaged at 30 hpf (Fig 5A) to determine the contribution of the transplanted cells. Those embryos with sizeable contribution to maxillary neural crest cells were then grown to 4 dpf and stained for cartilage and bone (Fig 5B, n = 10). The neurocrania were flat mounted and the trabeculae were scored as above. A Mann-Whitney test demonstrated that the presence of wild type neural crest cells significantly restored the trabeculae (Fig 5C, p<0.05). Of the 10 transplanted embryos six were chimeric on the left and four were

| Table 1. Contralateral score. |
|-----------------------------|
| Right side | 0 | 1 | 2 | 3 |
| Left side |
| 0 | 2 | 2 | 0 | 0 |
| 1 | 7 | 12 | 3 | 0 |
| 2 | 1 | 6 | 4 | 0 |
| 3 | 0 | 0 | 0 | 0 |
chimeric on the right. While there is often phenotypic variability within an individual gata3 mutant, we find that the phenotypic score on the transplanted side was always improved relative to the non-transplanted side. To determine if donor neural crest cells contribute to the rescued trabeculae, we transplanted cells from ubi:Switch (ubiquitously labeling cells green in the

Fig 3. Dynamic expression of gata3 in maxillary neural crest cells. (A-D) Lateral views, anterior to the left, of gata3 in situ hybridization. (A) At 22 hpf, gata3 is expressed in the head in brain (black arrow), ear (e) and what is likely to be endoderm (*). (B) By 24 hpf, gata3 expression domains include cranial neural crest cells, which contain the trabeculae precursors (white arrowheads), the brain and neurons. (C) At 26 hpf expression continues in all previously expressing tissues. The white arrowheads indicate the cranial neural crest cells that give rise to trabeculae. (D) At 48 hpf expression begins to be down regulated in the developing palate. (E-G) RNA Scope V2 whole mount in situ hybridization at 36 hpf; white arrowheads bracket the trabeculae precursors. (E) Expression of pdgfra in cranial neural crest cells. (F) Expression of gata3 in maxillary cranial neural crest cells that will give rise to trabeculae. (G) The overlap of pdgfra and gata3 in trabeculae precursors.

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absence of Cre) into sox10:mCherry hosts injected with gata3 morpholino. We find that these transplanted cells are capable of contributing to the trabeculae and that the trabeculae is rescued on the transplanted side (Fig 5F–5H). These results support a model in which neural crest cells require the function of gata3 for proper trabeculae morphogenesis. However, it remains possible that gata3 is not specifically required within the progenitors of the trabeculae, a genetic fate map of the descendants from these gata3-positive cells will aid in understanding.

**Gata3 functions downstream of Bmp**

We next asked how Gata3 may function in the development of such a specific part of the skull and why the phenotype can be so highly variable. While blocking Bmp signaling disrupts multiple aspects of craniofacial development, similar trabeculae defects can be generated via a
dominant negative form of bmp1a [35]. Furthermore, Smad1/5 binds upstream of Gata3 in mouse neural crest cells [30]. These results suggest that gata3 may be a Bmp target in the maxillary neural crest. We performed in situ hybridization with gata3 riboprobe in 36 hpf smad5-/- and wild type embryos (Fig 6). We find a striking reduction in the maxillary expression of gata3 when compared to the wild type sibling (Fig 6, arrowheads). Interestingly, brain and ear expression are mostly unaffected (Fig 6). These data demonstrate that the neural crest expression of gata3 requires Bmp signaling. Collectively, these findings suggest an epistatic relationship in which Bmp signaling to the neural crest is upstream of gata3. This predicts that maxillary neural crest will be Bmp responsive. We detected Bmp responsive cells using BRE:d2EGFP transgenics, in which a destabilized form of GFP is expressed from a Bmp response element. We crossed this line to the sox10:mRFP line, allowing us to visualize the overlap of Bmp signaling within the neural crest. Double-labeled embryos were imaged at 24 hpf, the time when gata3 function is required (Fig 6E–6G). At this time, maxillary neural crest cells display a robust bmp response (Fig 6E and 6G, arrows). Furthermore, using hsp:DN-Bmpr1 transgenics, we demonstrate that the maxillary expression of gata3 requires Bmp signaling at this time (Fig 6C and 6D). These data support a model in which Bmp signaling is upstream of gata3, driving palate development. This epistatic relationship predicts that forced gata3 expression should partially compensate for the loss of Bmp signaling. We generated hsp:GATA3-EGFP;hsp:DN-Bmpr1a double transgenics to simultaneously express a dominant negative Bmp receptor and GATA3. We heat shocked embryos between 24 to 28 hpf, time points important for gata3 function, and grew all fish to 4 dpf when they were stained for cartilage and bone then scored for phenotypes (Fig 7). We find that at each time point, co-expression of GATA3 and the DN-Bmp receptor improved the trabeculae phenotype relative to embryos only expressing DN-Bmp receptor (Fig 7B and 7D). The complete list of statistics is in Table 3. As controls for potential non-specific interactions based on the use of two hsp promoters, we examined the phenotypes of hsp:DNBmpR1a;hsp:Gal4 double transgenics. Phenotypes in these embryos mirror those found in hsp:DNBmpR1a single transgenics (S1 Fig). Whole larve images of 5 dpf heat shocked, control embryos and gata3 mutant stained for cartilage and bone are also in S1 Fig. Additionally, we characterized fluorescent intensity at the cell membrane in hsp:DNBmpR1a and hsp:DNBmpR1a:hsp:GATA3-EGFP. We imaged periderm cells due to their large, flat morphology making it easy to distinguish cell membrane (DNBmpR1a) and nuclear (GATA3) labeling. We find no difference in fluorescent intensity between the single and double

| Controls | x  | n  | S.E.M. | P-value |
|----------|----|----|--------|---------|
| gata3-/- | 1.1| 84 |        |         |
| hsp:GATA3-EGFP;gata3-/- no heat shock | 1.0| 124| 0.1118 |         |
| Hpf of heat shock hsp:GATA3-EGFP;gata3-/- |  |
| 18       | 1.0| 12 | 0.2441 | n.s.    |
| 22       | 1.3| 53 | 0.1388 | n.s.    |
| 24       | 1.9| 73 | 0.1266 | <0.0001 |
| 26       | 1.6| 36 | 0.1576 | 0.0245  |
| 28       | 1.3| 80 | 0.1236 | n.s.    |
| 30       | 1.6| 60 | 0.1337 | 0.0068  |
| 32       | 1.3| 42 | 0.1495 | n.s.    |
| 48       | 1.2| 36 | 0.1576 | n.s.    |

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transgenics (S2 Fig). Taken together our results place gata3 downstream of Bmp signaling with both being required for proper trabeculae morphogenesis.

The Bmp pathway has many targets in the neural crest. One possible explanation for the phenotypic plasticity in gata3 mutants is variable compensation by other Bmp targets. This model predicts that attenuation of Bmp signaling in a gata3 loss-of-function embryo will cause more severe phenotypes. To test this, we injected a gata3 morpholino that faithfully recapitulated the phenotypes observed in our gata3

morphploids null mutant into embryos heterozygous for smad5. However, the phenotypes in gata3 morpholino-injected wild type and smad5 heterozygous embryos were identical (p = 0.152, wild type mean = 0.87, S.E.M. = 0.1 n = 52; heterozygotes mean = 0.67, S.E.M. = 0.09, n = 72). While these findings do not rule out the possibility that other Bmp targets modulate the phenotype of gata3 mutants, they suggest that gata3 is a critical target.
Fig 6. Bmp signaling to maxillary neural crest regulates the expression of gata3. (A-D) Lateral views with anterior to the left of 36 hpf gata3 in situ hybridization black arrows point to maxillary crest that will give rise to trabeculae; white arrows mark the ear. (A-B) Embryos from a smad5 clutch. (A) Wild type embryo showing strong expression of gata3 in maxillary trabeculae precursors. (B) Mutant embryo showing loss of gata3 expression in trabeculae precursors. (C-D) Embryos from a hsp:DN-Bmpr1a clutch. (C) No heat shock embryo arrowhead indicates gata3 expression in maxillary precursors. (D) Embryo heat shocked at 24 hpf arrowhead indicates loss of gata3 expression in maxillary CNCC. (E-G) Confocal images of Bmp responsive cells (green, cytoplasmic) and CNCC (red, membrane tagged), lateral views with anterior to the left at 24 hpf. Arrow points to Bmp responsive maxillary neural crest cells.

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Shh signaling modulates the phenotypes in gata3 loss-of-function embryos

In our previous analyses, we found that Shh signaling was critical for development of the zebrafish palate [2]. In those hypomorphic shha mutants that retained the palate we often found

Table 3. Statistics for rescue of bmp.

| Hpf of heat shock | \( \bar{x} \)  | n  | S.E.M.   | P-value   |
|------------------|-------|----|---------|----------|
| 24               | \( \text{hsp:DN-BmpR1} \)   | 0.79| 38      | 0.1468   | 0.0007   |
|                  | \( \text{hsp:DN-BmpR1;hsp:GATA3-EGFP} \) | 1.55| 44      | 0.1734   |          |
| 26               | \( \text{hsp:DN-BmpR1} \)   | 1.53| 68      | 0.1397   | 0.0082   |
|                  | \( \text{hsp:DN-BmpR1;hsp:GATA3-EGFP} \) | 2.17| 42      | 0.1895   |          |
| 28               | \( \text{hsp:DN-BmpR1} \)   | 2.32| 64      | 0.1406   | 0.0131   |
|                  | \( \text{hsp:DN-BmpR1;hsp:GATA3-EGFP} \) | 2.84| 52      | 0.0841   |          |

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disrupted stacking of chondrocytes in the trabeculae (S3 Fig). While this mutant also has characteristic midline defects, here we are interested in the trabeculae defects. This similar phenotype in the trabeculae suggests that the Shh pathway may be acting in concert with Gata3 to promote palatogenesis. We took advantage of our inbred gata3\(^{b1075}\) mutant lines selected for severe and mild phenotypes to test the effects of modulating Shh signaling on gata3 phenotypes.

If Shh promotes palatal development independent of Gata3 function, then elevation of Shh signaling should ameliorate the phenotypes of gata3\(^{-/-}\) mutants. We elevated Shh signaling using the Smoothened agonist SAG. We found that a treatment with 12.5 uM SAG did not alter wild type development but improved the phenotypes of gata3\(^{-/-}\) mutants in the severe background (Fig 8). While mutants in the severe genetic background typically lack trabeculae (Fig 8A and 8C), treatment with 12.5 uM SAG resulted in a significant improvement in the trabeculae score (Fig 8B and 8C). Thus, consistent with our model, elevating Shh signaling is able to partially compensate for the loss of gata3.

This model also predicts that reduction in Shh signaling will exacerbate the phenotypes in our mild gata3 mutants. We used 12.5 uM cyclopamine to reduce Shh signaling to a level that doesn’t disrupt trabeculae development in either of the genetic backgrounds used in these analyses (n = 26 WIK; n = 28, EK), we note that typical zebrafish doses to disrupt craniofacial development at this age range between 50 and 100 uM. We find that this low dose of...
cyclopamine is capable of exacerbating the palatal phenotypes in mild gata3 mutants relative to vehicle-treated mutants (Fig 8). Collectively, these findings demonstrate that the overall level of Shh signaling is capable of modifying, in both directions, the phenotypes of gata3 mutants.

Many human diseases, including HDR, are due to haploinsufficiency. Based on our findings with Shh, we sought to determine if a low dose of cyclopamine could cause haploinsufficiency in the severe gata3 mutant background. In vehicle-exposed embryos, we find that all gata3 heterozygotes develop normally. (C-D) Cyclopamine-treated embryos. (C) Wild type embryo showing normal stacking and fusion of trabeculae (n = 22/22). (D) Heterozygote embryo displaying complete loss of trabeculae with severe clefts (n = 6/66).

These findings suggest that there may be cross talk between Gata3 and Shh. Higher doses of SAG or cyclopamine do not alter the expression of gata3 (S4 Fig). In order to determine if alterations in Shh signaling modulates the expressivity of gata3 mutants, we turned to quantitative fluorescent in situ hybridization across gata3 mutant backgrounds. We found that the levels of ptch2 were equivalent in wild type embryos across the two genetic backgrounds (Fig 10). Interestingly, relative to the respective wild type embryos, “mild” mutants significantly upregulate the expression of ptch2, while “severe” mutants do not (Fig 10, see S5 Fig for...
zoomed out views). Collectively, these findings strongly suggest that a compensatory upregulation of Hh signaling can lessen the phenotypic outcome of loss of Gata3 function.

**Discussion**

**Gata3 functions downstream of Bmp signaling in neural crest precursors of the palatal skeleton**

Despite its known involvement in palatal development, the role of Gata3 in this process is uncharacterized. We have found that gata3 is required for proper development of a region of the zebrafish palatal skeleton, the trabeculae. The expression of gata3 localizes to regions that contribute to the trabeculae [2,3,6]. Consistent with its expression pattern, we find that neural crest cells require Gata3 function for palatogenesis. While detailed expression analyses during palatal development in other species have not been performed, Gata3 is strongly expressed in maxillary/frontonasal regions of mouse and chicken embryos [29,30,36]. Thus, Gata3 is likely to have evolutionarily conserved functions in palatal development. Analyses in conditional mouse mutants would aid in characterization of these conserved functions.

The regulation of Gata3 expression also appears evolutionarily conserved. Using BRE:d2GFP transgenics, we demonstrate that the neural crest cells that express gata3 receive Bmp signaling. The expression of gata3 in the neural crest, but not other tissues, is lost when Bmp signaling is reduced in hypomorphic smad5 mutants, demonstrating that Bmp signaling is necessary for gata3 expression. While their analyses focused on mandibular development, Bonilla-Claudio and colleagues demonstrate that over action of Bmp results in the expansion of Gata3 expression in the frontonasal and maxillary prominences [30]. Interestingly, the Drosophila homologue of Bmp (decapentaplegic) induces Gata (pannier) expression during development of the notum [37]. Thus, Bmp-Gata signaling may have arisen very early in animal evolution.
It is likely that Bmp signaling directly activates Gata3 expression. The time windows when forced expression of human GATA3 can partially rescue gata3 mutants and DN-Bmpr1a transgenics are largely overlapping. Work in the mouse mandible has demonstrated that Smad1/5 binds a region upstream of Gata3 [30]. While these studies did not assess Smad1/5 binding in maxillary neural crest, these collective findings prompt similar analyses in maxillary crest.

**Interactions between Bmp and Shh signaling modulate variability**

Three general mechanisms could explain the variation in gata3 mutant phenotypes: 1) Residual gene function in the mutant, 2) partial compensation via other Bmp target genes and 3) phenotypic modulation via a different signaling pathway.

**Residual gene function.** Previously, we demonstrated that a point mutation of gata3 generated phenotypes that were highly variable dependent upon genetic background [13]. This b1075 allele mutated a Cysteine that is homologous to one disrupted in HDR syndrome and required for coordinating the second zinc finger necessary for DNA binding [13,32]. The human GATA3 mutation (Cys318Arg) was hypothesized to be a null based on *in vitro* data [32]. However, the variability in b1075 mutants left open the possibility that these missense mutants retained some functionality *in vivo*.

Our results here strongly support the model in which the human Cys318Arg mutation is a null. The au42 allele generates the same range of phenotypes as does b1075, although we have not generated inbred lines within different genetic backgrounds to test for modulation by genetic background. The au42 mRNA encodes a protein predicted to terminate before the zinc finger domains, which are necessary for Gata3 function. Thus, the variability in gata3 mutant phenotypes is unlikely to be due to residual gene function.

**Partial compensation.** Most major signaling pathways have a multitude of target genes that behave in networks to drive development. The Bmp pathway is no exception with a growing number of characterized targets in craniofacial development generally [30,38,39] and palatogenesis more specifically [4]. One or more of these targets could be partially redundant with gata3. For instance, both Gata3 and Gata2 are regulated by Bmp during mandibular development [30] and these same two genes are partially redundant during trophoblast development [40]. This model predicts that attenuation of Bmp signaling in gata3 loss-of-function embryos will worsen the resulting phenotypes. However, we did not detect such an interaction. While we cannot rule out potential redundancy between Bmp targets in the generation of the trabeculae phenotypes, it would appear that this is not the major cause of phenotypic variability in gata3 mutants.

**Interaction between pathways.** A wide array of signaling pathways are involved in palatogenesis, with the Bmp, Fgf and Shh pathways being particularly important [4]. Cross talk between the Bmp and Shh pathways has been demonstrated in palate development [41]. In addition to their roles in palatogenesis, interaction between the Bmp and Shh pathways appear to mediate facial shape [42]. We and others have shown that both of these two pathways are crucial for palatogenesis in zebrafish [2,3,6,35]. Interestingly, Gata6 has been shown to bind Gli transcription factors [33]. Cross-talk between these pathways provides mechanisms for interactions to generate variability. Our findings do not rule out the involvement of other pathways in modulating the phenotypes in gata3 mutants. Indeed, we have previously demonstrated that altering Hsp90 activity can change the phenotypes in these mutants [13]. While the mechanism of this interaction is unknown, Hsp90 has a large number of client proteins involved in signaling [43]. However, our results clearly demonstrate that interactions between Shh and the Bmp-Gata3 pathway modify the phenotypes generated in gata3 mutants.
Implications for variation in human birth defects

Our current findings implicate a Bmp-Gata3 signaling pathway in the genesis of microsomia and HDR-related craniofacial defects (including palatal defects), both highly variable craniofacial defects. There are no known causative variants for craniofacial microsomia in humans. However, GATA3 is associated with microsomia, a disease that is thought to be caused by gene-environment interactions [23–25]. Our results provide strong evidence that GATA3 is responsible for craniofacial microsomia and likewise implicate Bmp pathway members in the etiology of microsomia.

Phenotypic variation is a common theme in birth defects. Such variation is often attributed to genetic background and gene-environment interactions. Our results demonstrate that both of these processes can alter the phenotypes of gata3 mutant. We previously demonstrated that genetic background strongly modified the phenotype resulting from gata3 mutation [13]. Here we demonstrate that this difference in expressivity is modulated via the level of Shh signaling. Interestingly, we find that gata3 mutants in the “mild” genetic background have a compensatory upregulation in Shh signaling. Our inhibitor and agonist studies demonstrate that altering Shh signaling modifies the phenotypic outcomes in gata3 mutants. Therefore, the upregulation of Shh signaling in the “mild” background is likely to be an important contributor to the less severe phenotype. The precise nature of the compensatory upregulation of Shh signaling is unknown, but will provide important insight into developmental robustness.

Our finding that Shh signaling can modulate the phenotypes in gata3 mutants has important implications for understanding the potential for gene-environment interactions in birth defects. Haploinsufficiency for Shh signaling pathway members underlie holoprosencephaly, another disease that is thought to be caused by gene-environment interactions [44–46]. A growing body of research demonstrates that the Shh pathway is extremely sensitive to environmental attenuation. For instance, the common chemical synergist piperonyl butoxide (PBO) and many dietary molecules such as tomatidine and solanidine can inhibit Shh signaling [47–49]. Microsomia is thought to have a large environmental component [26]. The interaction between cyclopamine and Gata3 implicate similar influences, with environmentally relevant molecules such as PBO in the genesis of microsomia and HDR syndrome. Collectively, these results suggest that alterations to Gata3 and its associated regulatory network destabilizes craniofacial development and that the overall level of Shh signaling modifies the response to this destabilization.

Materials and methods

Ethics statement

For the animal experiments, all procedures were performed according to an approved IACUC protocol (AUP00002018) at UT Austin.

Fish care and fish lines

Zebrafish (Danio rerio) were raised according to [50,51] and were staged as previously described [52]. The following transgenic lines were used: BmpRE::AAV.mlpl:d2GFPpwm30 [53], hsp70l:dnXla.BmpR1aGFPl30 [54], Tg(fli1:EGFP)v1 [55], Tg(sox10:mRFP)m2 [56], Tg3.5ub:LOXP-EGFP-LOXP-mCherry[57], Tg(hsp70l:Gal4) [58] and are referred to as BRE:d2GFP, hsp:DN-BmpR1, fli1:EGFP, sox10:mRFP, ubi:Switch and hsp:Gal4 for clarity. The smad55100 allele is described [6]. The gata3b1075 allele is described; the “mild” background was generated by outcrossing to WIK and the “severe” background was generated in a cross to fli1:EGFP (in the EK background)[13]. Wild type stocks were AB unless otherwise noted.
Generation and genotyping of gata3 au42 CRISPR line

We utilized ZiFiT Targeter (http://zifit.partners.org/ZiFiT/) to identify gRNA binding sites for gata3. With these target sites, we made gata3 gRNA via MEGAscript T7 Kit and Cas9 mRNA via T3 mMessage Kit (Invitrogen) using a described protocol [59]. Embryos were injected with a 2 nl bolus of a cocktail containing: 100 ng/ul Cas9 mRNA, 50 ng/ul gata3 gRNA in water and phenol red (to visualize the injection).

Embryos were genotyped using either Restriction fragment length polymorphism (RFLP) or High-resolution melt (HRM) analysis. For RFLP we used forward 5’-GGTATGACGAATCCCACAACAGAC-3’ and reverse 5’-AAGAGGACCCACCTATCAGGCTAC3’ primers. Digestion with NlaIV results in a 531 bp mutant band and 394 and 139 wild type fragments. Primers for HRM analyses were forward GGCAAATCTATCGGCCTCA and reverse GGACAGCGAGGAAG. The resulting product is 129 bp.

Generation of Tg(hsp70:GATA3-EGFP) au34

We obtained full-length human GATA3 in pDonor from human ORFeome Version1.1 (cat# OHS1770-9380128, genbank access #CV025706). Using the Tol2 kit [60], we recombed the GATA3 MEC vector, the P5E-hsp70 (#222, 5’ entry clone), P3E-EGFP (#366, 3’ clone) and destination vector pDest cy2 (#395) to make a 10267 bp vector. A mix of 100 ng plasmid, 155 ng transposase RNA and phenol red were injected as a 3 nl bolus into AB fish and screened for glowing hearts. We refer to the recovered transgenic line as hsp:GATA3-EGFP for clarity.

Tissue labeling and imaging and analyses

Cartilage and bone staining was done as previously described [61]. A modification of a published protocol [52] was used to visualize the dissected neurocranium. In particular, the brain and overlying ectoderm was left intact to hold cartilages and bones in their context. The gata3 riboprobe is described [62]. RNA in situ hybridization was performed according to published protocols [63]. Fish used for in situ analyses were raised in 0.0015% PTU (1-phenyl 2-thiourea) to inhibit melanin production. Activation of all heat shock lines was performed by placement in a 39˚C water bath for 50 minutes. Fluorescent whole mount in situ were performed using RNA Scope version 2. ACD Biology designed probes to gata3, ptc2 and pdgfra. We modified the existing version 2 protocol and the whole mount zebrafish technical note outlined by ACD Biology. We also integrated published recommendations [64]. A complete protocol is outlined (S6 Fig).

Colorometric in situ hybridizations and cartilage/bone stained embryos were imaged on a Zeiss Axioimager. Confocal images of embryos were collected on a Zeiss 710 microscope. All images were processed in Photoshop CS3. All statistics and graphs were generated in Prism versions 7.0–9.0.

For quantification of ptc2 expression, we used identical imaging settings for all embryos. We used a 40x water lens with a 2x digital zoom. For each embryo three individual z sections (4 um apart, a distance which prevented the same dot from being sampled across two z sections) were selected for counting dots of labeled ptc2 probe. For consistency, we selected a 5 um x 50 um region of maxillary neural crest cells immediately adjacent to the oral ectoderm and below the eye, known to contribute to the anterior neurocranium, for counting. The same region was selected across embryos using transmitted light initially and was verified as neural crest cells using pdgfra as a neural crest cell marker. The number of dots was manually counted. All counts were performed blinded to genotype.
Chimera analyses
We generated two types of genetic chimeras by neural crest transplantation from sox10:mRFP into gata3:au42;fl1:EGFP embryos and ubiquitously into gata3 LOF;sox10:mcherry as described [65]. For gata3 LOF embryos we injected 15ng of a gata3 morpholino as described [13]. The resulting embryos were imaged at 30 hours post fertilization (hpf) to access contribution of donor cells to the maxillary region of the first arch. To characterize ubiquitously neural crest cell incorporation into the neurocranial the viserocranium was dissected away and the neurocranium was imaged at 4dpf. Neurocrania were then stained with Alcian Blue and Alizarin Red.

Chemical treatments
Cyclopamine and SAG treatments were performed as described elsewhere [66,67]. Both cyclopamine and SAG were used at 12.5μM, a dose that did not disrupt development in wild type embryos. Embryos were treated from 24 to 30 hpf. Embryos were grown to 5 dpf and stained for cartilage and bone.

Supporting information
S1 Fig. Cartilage and bone stained 5 dpf larvae. (A-D) Representative embryos of each genotype were imaged laterally with head to the left. Blue and red staining indicate cartilage and bone respectively (A) non-heat shocked wild type zebrafish. (B) Heat shocked wild type fish have normal morphology. (C) Heat shocked hsp:DN-BmpR1a fish show some disruption to normal development, cardiac edema and alterations to the face. (D) Heat shocked hsp:DN-BmpR1a;hsp:GATA3-GFP fish appear normal. (E) Heat shocked hsp:DN-BmpR1a;hsp:Gal4 show similar disruptions to development as in C, providing genetic evidence that a double heat shock transgenic does not interfere with transgenic expression off of the heat shock promoter. (F) Overall, gata3 mutants have normal morphology. The image is of a gata3:au42 mutant.

S2 Fig. Double heat shock line does not diminish EGFP signal. (A-B) Confocal single-z images of embryos at 30 hpf, embryos were heat shocked at 24 hpf. Representative images of (A) a hsp:DN-BmpR1embryo and (B) a hsp:DN-BmpR1;hsp:GATA3-EGFP embryo, arrows indicate cell membrane expression of DN-BmpR1-EGFP. Intensity values for hsp:DN-BmpR1 (n = 11, mean = 8663, standard deviation = 1585, n = 11) were not statistically different from the intensity values for hsp:DN-BmpR1;hsp:GATA3-EGFP (mean = 10583, standard deviation = 3463, n = 11), p-value = 0.1101 by t-test.

S3 Fig. Trabeculae defects in a shh hypomorph. Flat mount of a 5dpf shh:ta252/ta252 mutant neurocranium anterior to the left. Arrow is pointing to disrupted stacking of trabeculae cells.

S4 Fig. Shh does not affect the expression of gata3. (A-D) Lateral views of embryos at 36 hpf showing normal expression of gata3 regardless of treatment. (A) Embryo treated with DMSO as a SAG treatment control. (B) Cyclopamine treatment control embryo treated with vehicle ETOH. (D) Embryo treated with 25uM SAG (C). 25 uM cyclopamine treated embryo.

S5 Fig. First arch images of ptch2 expression. Single-z images of DIC and patched expression in the first pharyngeal arch. Arrows indicate maxillary neural crest. Eye is indicated by e.
S6 Fig. Whole mount RNA Scope version 2 fluorescent in situ protocol.

(DOCX)

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Author Contributions
Conceptualization: Mary E. Swartz, Johann K. Eberhart.
Data curation: Mary E. Swartz.
Formal analysis: Mary E. Swartz.
Funding acquisition: Johann K. Eberhart.
Investigation: Mary E. Swartz, C. Ben Lovely.
Methodology: Mary E. Swartz, Johann K. Eberhart.
Project administration: Johann K. Eberhart.
Resources: Johann K. Eberhart.
Supervision: Johann K. Eberhart.
Validation: Mary E. Swartz.
Visualization: Mary E. Swartz.
Writing – original draft: Mary E. Swartz.
Writing – review & editing: Mary E. Swartz, C. Ben Lovely, Johann K. Eberhart.

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