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Role of *mef2ca* in developmental buffering of the zebrafish larval hyoid dermal skeleton

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ABSTRACT

Phenotypic robustness requires a process of developmental buffering that is largely not understood, but which can be disrupted by mutations. Here we show that in *mef2ca*^{b1086} loss of function mutant embryos and early larvae, development of craniofacial hyoid bones, the opercle (Op) and branchiostegal ray (BR), becomes remarkably unstable; the large magnitude of the instability serves as a positive attribute to learn about features of this developmental buffering. The OpBR mutant phenotype variably includes bone expansion and fusion, Op duplication, and BR homeosis. Formation of a novel bone strut, or a bone bridge connecting the Op and BR together occurs frequently. We find no evidence that the phenotypic stability in the wild type is provided by redundancy between *mef2ca* and its co-ortholog *mef2cb*, or that it is related to the selector (homeotic) gene function of *mef2ca*. Changes in dorsal–ventral patterning of the hyoid arch also might not contribute to phenotypic instability in mutants. However, subsequent development of the bone lineage itself, including osteoblast differentiation and morphogenetic outgrowth, shows marked variation. Hence, steps along the developmental trajectory appear differentially sensitive to the loss of buffering, providing focus for the future study.

Introduction

How is an intricate, complex and integrated morphological pattern, such as we see for bones of the vertebrate skull, reliably achieved during development? Leading studies and ideas about phenotypic stability and its regulation derive from Schmalhausen and particularly from Waddington, who proposed that development is 'canalized', or buffered against the perturbing effects of genetic mutation and environmental disturbances (Dworkin, 2005; Flatt, 2005; Schmalhausen, 1949; Waddington, 1957). Waddington depicted canalization graphically in an icon that has become well known – canalization is a sloping landscape of hills and valleys. Development is a ball rolling down the landscape, and genes regulating canalization sculpt the contours guiding the pathway of the ball – elevating the hills, deepening the valleys, and hence stabilizing the developmental trajectory. Waddington (1957) also considered that buffering might not effect just the influences of genetic mutation and environmental irregularity but also the 'inherent noisiness of a developmental pathway'. We understand developmental noise to be the product of nondeterministic fluctuations in molecular mechanisms that underlie development, for example stochastic variation in the collisions between small numbers of regulatory macromolecules that need to bind together in order to serve as effectors of progression along developmental pathways (Dongen, 2006; Polak, 2003). Because of noise, development might fail to reach a specific 'target' phenotype even in the absence of genetic and environmental variation (Polak, 2003). Such a failure, a change in morphology due to random noise, is termed developmental instability (Polak, 2003). Later authors have debated whether buffering the effects of mutation and environment ('canalization'), and buffering developmental noise are the same thing (Breuker et al., 2006; Debat et al., 2009; Hallgrimsson et al., 2002; Nijhout and Davidowitz, 2003), and the issue remains controversial.

Buffering can break down, resulting in increased phenotypic variation; indeed, the initial motivation for the canalization concept was to explain the increased variation commonly observed in mutants (Waddington, 1942). Apparent loss of buffering characterizes hyoid arch dermal bone development in zebrafish

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Endothelin1 (Edn1) pathway mutants (Kimmel et al., 2003). Edn1 provides an extracellular signal which functions along with Notch and BMP signaling in craniofacial development (Kimmel et al., 2007; Medeiros and Crump, 2012). In *edn1* mutants the hyoid arch dermal bones of the early larva, the opercle (Op) and branchiostegal ray #3 (BR), show remarkably contrasting phenotypes (Kimmel et al., 2003) (for developmental anatomy see Eames et al. (2013)). In some mutants both the BR and Op are missing, but in others the Op is enlarged. Furthermore, sometimes Op loss and Op expansion occur together on opposite sides of the same mutant (Kimmel et al., 2003), suggesting developmental instability. One interpretation of these findings is that Edn1 signaling, in a complex manner, normally regulates both activation and repression of OpBR development: loss of one or the other downstream function – activator or repressor – variably shows up separately in the *edn1* mutant.

Our craniofacial genetic screen yielded an allele of an Edn1-pathway gene that is particularly useful for understanding the OpBR phenotype (Miller et al., 2007), and is the subject of this paper. This mutation, *mef2ca*^{b1086} (hereafter *mef2ca*$^{-}$), likely causes complete loss of function of a MADS box-containing transcription factor encoding gene critical for skeletal development, *Mef2c* (Arnold et al., 2007; Miller et al., 2007; Verzi et al., 2007). *mef2ca* functions downstream of *edn1*, as revealed by double-mutant and other analyses (Miller et al., 2007). As we show here, in the zebrafish strain AB genetic background in which the *b1086* mutant allele was identified, the phenotype is highly variable in expressivity of the OpBR phenotype, which facilitates study and understanding of the basis of the variation. Furthermore, in extreme examples the BR resembles the Op in size and shape, suggesting the phenotype is homeotic (Miller et al., 2007). This hypothesis that *mef2ca* functions as a homeotic selector gene is in keeping with our current understanding of the developmental role of the gene network activated by Edn1 signaling. That is, in response to mutational loss of the Edn1 signal that is normally expressed in the ventral part of the arch (Miller et al., 2000), the more ventral BR might homeotically transform to express features of the more dorsal Op.

Here we further characterize the OpBR phenotype in *mef2ca* mutants, examining in particular what developmental steps appear to be associated with increased phenotypic variation. Our results show that developmental instability increases dramatically in the mutants. Phenotypic stability in the wild type is unlikely to be provided by redundancy between *mef2ca* and its co-ortholog *mef2cb*. Developmental analyses provide no evidence that disrupted early pattern specification or homeotic selector function play any direct role in the increased variation in *mef2ca* mutants. On the other hand we found marked variation in the location and time of appearance of ectopic osteoblasts that contribute to the expanded bone, and variation in subsequent morphogenetic bone outgrowth, including variable occurrence of a novel pattern of bone formation. We propose that loss of buffering is manifested in these relatively downstream developmental processes.

Materials and methods

Zebrafish lines

Zebrafish were reared according to standard protocols (Westerfield, 2007) and staged as previously described (Kimmel et al., 1995; Parichy et al., 2009). All experiments were approved by the University of Oregon Institutional Animal Care and Use Committee (IACUC). Zebrafish lines, including PCR-genotyping of mutants, were as described: *mef2ca*^{b1086} (Miller et al., 2007), *mef2cb*^{fh288} (Hinits et al., 2012), *furina*^{tg419} (Walker et al., 2006), *Tg*(*sp7*:*EGFP*)*b1212* (hereafter *sp7*:*EGFP*) (DeLaurier et al., 2010), *dlx5a*^{j107Et} (hereafter *dlx5a*:*EGFP*) (Talbot et al., 2010) and *trps1*^{J1271aGt} (hereafter *trps1*:*EGFP*) (Talbot et al., 2010).

Tissue labeling

Alcian Blue–Alizarin Red stains on fixed animals and vital staining with Alizarin Red were performed as previously described (Kimmel et al., 2010; Walker and Kimmel, 2007). Two-color fluorescent whole mount in situ hybridization was carried out as described (Talbot et al., 2010) Probes were as described: *ihha* (Avaron et al., 2006), *sp7* (DeLaurier et al., 2010) to label early matrix-secreting osteoblasts (Huycke et al., 2012; Li et al., 2009), and *runx2a* (Flores et al., 2004) to label preosteoblasts (Li et al., 2009).

Microscopy

Skeletal preparations were imaged on a Zeiss Axiophot 2. Static confocal images, either of live preparations or in situ preparations, were captured on either a Zeiss LSM 5 Pa confocal or a Leica SD6000 spinning disk confocal with the Borealis illumination technology. Images were assembled in ImageJ and Photoshop with any adjustments applied to all panels. For time-lapse recordings, animals were imaged on the spinning disk confocal as described (Huycke et al., 2012). To avoid photodamage, intervals were at least 25 min, and duration of the recordings was 24 h or less (Jemielita et al., 2012). Movies were assembled using Metamorph (Molecular Devices) and ImageJ.

Bone size analysis

Bone size analysis used a large cross of 6 dpf (days postfertilization) larvae obtained from single pair of *mef2ca* heterozygotes on the strain AB background. The sizes were obtained in duplicate from digitized outlines in ImageJ, and included the Op and BR added together when two separate bones were present (as in wild types and a subset of the mutants). Sizes are reported as area$^{1/2}$. Analyses of fluctuating asymmetry, quantified as the absolute difference between bone size on the left and right of single individuals, followed published guidelines (Palmer and Strobeck, 2003) with the replicates used to estimate measurement error. Statistical procedures were implemented in JMP (SAS Institute, Inc.).

Results

Extraordinary variation in mutant hyoid bone phenotype

The *mef2ca* mutant phenotype prominently includes expansion and shape deformation of OpBR bones in the larval hyoid arch. Bone expansion in mutants signals that the wild-type gene functions as a repressor of bone development (Miller et al., 2007). Whereas the mutants can be scored reliably by their cartilage phenotypes, OpBR expressivity is variable among clutches (Supplementary Table 1). Here we focus on within-clutch variation, examined in Figs. 1 and 2 in a set of full siblings. One is immediately struck by the remarkable OpBR phenotypic variation. The ectopic bone may have the appearance of a mirror-image duplicated opercle (Fig. 1C, Op′). An ectopic bony strut (Fig. 1D, s), or a bone bridge between the Op and BR is frequently present (Fig. 1J, b). In other examples a BR may be unrecognized, likely missing (Fig. 1E). Shape variation among mutants appears dramatically greater than in the wild type.