doi:10.1017/S1431927613002067
Microsc. Microanal. 19 (Suppl 2), 2013

Hand1 Dimer Regulation within the Medial Cranial Neural Crest is Required For Tissue Fusion and Pallet Formation

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Congenital craniofacial defects (CCDs) represent 1 in 500-700 live human births according to World Health Organization calculations. CCDs affect the ability of newborns to eat, to breath, and dramatically increases incidence of infections. Later in life, cleft lip/pallet impairs the ability to speak and the ability socially integrate into society is also compromised. CCDs are often encountered with other congenital defects such as cardiac abnormalities as the cell lineage causative of CCDs: the neural crest (NC) play critical roles in both craniofacial and cardiac development. Clinical treatment of CCDs requires surgical intervention where many patients require multiple surgeries. Collectively, the high incidence of CCD in live births, their debilitating consequences, and a general lack of understanding of the molecular mechanisms that underlie CCDs, results in clinical treatments that are reactive. It is the lack of a molecular understanding of craniofacial morphogenesis that limits development of proactive screening and pharmacological treatment during fetal development.

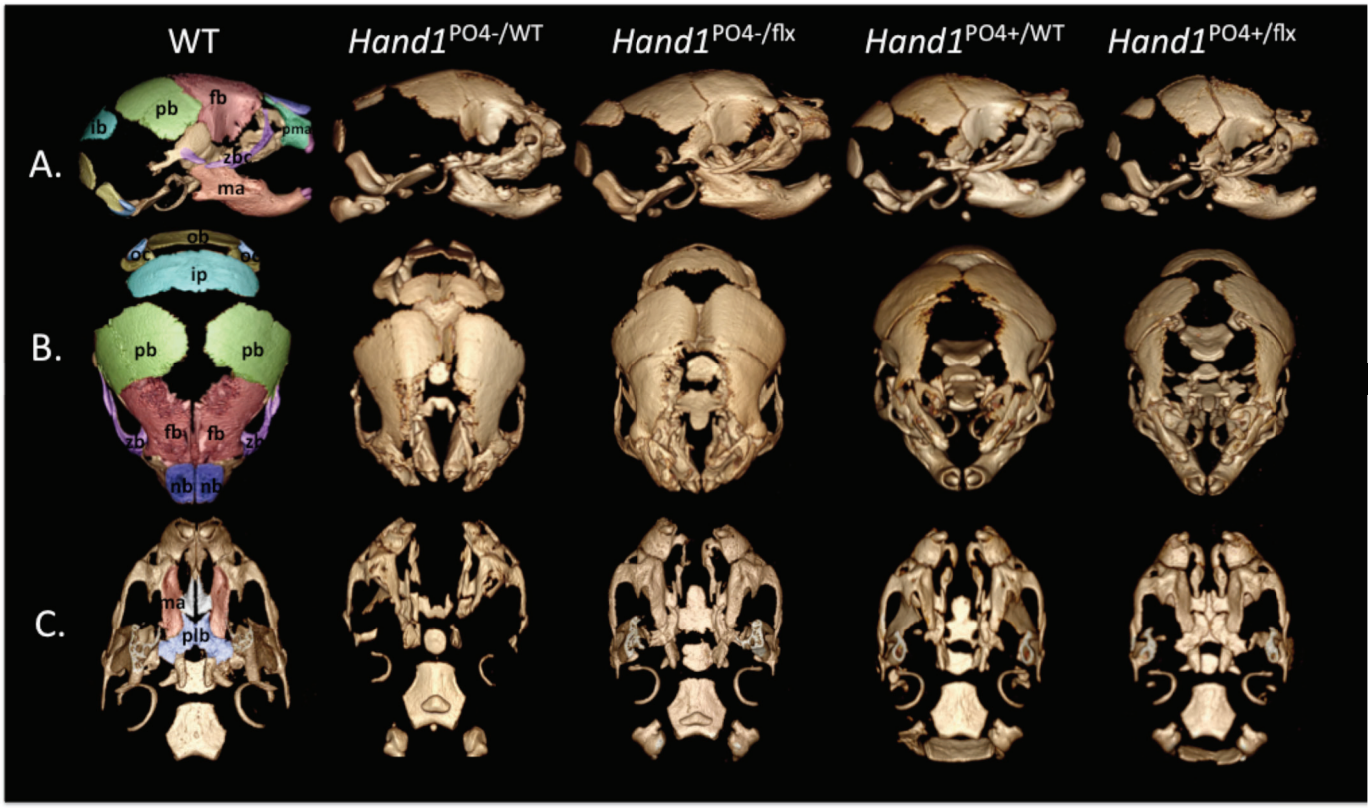
CCDs can have both a genetic and environmental component, which collectively determine clinical outcome. Members of the basic Helix-loop-Helix (bHLH) transcription factor family are established as critical regulators of NC development and function. The Twist-family bHLH factors Twist1 and Hand2 are causative of the human craniofacial diseases Saethre Chotzen Syndrome (SCS) and conditional loss-of-function mouse model analysis reveals their role in NC cells is required for normal craniofacial development. Research reveals a sensitive gene dosage relationship amongst all Twist-family factors making study of this family of bHLH proteins essential for understanding the etiology of CCDs. This sensitive gene dosage relationship is in part based on the functional requirement of bHLH factors to form dimer complexes, which in turn modulate transcription. The Twist-family bHLH protein Hand1 is expressed within only the medial most NC of the forming face and the deletion of Hand1 from this tissue results in no significant phenotype. In contrast, two Hand1 alleles that disrupt a known phosphoregulation cascade (regulating bHLH dimerization) result in a severe mid-face cleft due to a lack of tissue growth and fusion in mice. These gain-of-function mutants disrupt the genetic distribution of bHLH factors within this medial domain of cranial NC cells, which our data now show is required for craniofacial formation.

Craniofacial development was evaluated using a desktop micro-computed tomography (μCT) machine (SkyScan 1172 high-resolution μCT; SkyScan, Kontich, Belgium) with a source voltage of 59 kV and a 6μm isotropic voxel size was used to assess *ex vivo* craniofacial malformations between WT, *Hand1*$^{PO4-/WT}$, *Hand1*$^{PO4-/flx}$, *Hand1*$^{PO4+/WT}$, *Hand1*$^{PO4+/flx}$ genotypes at P0 (**Figure 1 A-E**). This imaging technique allows us to generate 3-D craniofacial images of mineralized tissue to identify structural phenotypic differences between genotypes to support our histological findings. Each image represents a total of

1,000-1,5000 6μm contiguous slices that are reconstructed into a 3D model. 3D images were created using OsiriX imaging software.

Taken together, our results show severe disruption of craniofacial structures that can be observed as early as E10.5. Given that complete loss-of-function in Hand1 within the NC results in no obvious phenotype, we can conclude that altering the ability of Hand1 to properly choose its dimer partner disrupts the bHLH dimer pool within the medial most NC and this disruptions results in a failure of NC tissue outgrowth as a result of increase cell death within the forming pharyngeal arches at E9.5.

Figure 1. Micro-computed tomography craniofacial P0 images. **A. Sagittal view.** oc, occipital condyles; ob, occipital bone; ip, interparietal bone; pb, parietal bone; fb, frontal bone; zb, zygomatic bone; nb, nasal bone; pma, pre-maxilla; and ma, mandible**. B. Transverse view**. ob, occipital bone; oc, occipital condyle; ip, interparietal bone; pb, parietal bone; fb, frontal bone; zb, zygomatic bone, nb, nasal bone. **C. Palate.** ma, mandible; plb, palatine bone.



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[1] Warden et al., Bone, 54 (2013), 98-105.

[2] the authors acknowledge funding from the NIH-NIAMS R01-AR0567740 and NIH-NIAMS K01 AR054408-01A.

https://doi.org/10.1017/S1431927613002067 Published online by Cambridge University Press