Hand1 phosphoregulation within the distal arch neural crest is essential for craniofacial morphogenesis

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ABSTRACT

In this study we examine the consequences of altering Hand1 phosphoregulation in the developing neural crest cells (NCCs) of mice. Whereas Hand1 deletion in NCCs reveals a nonessential role for Hand1 in craniofacial development and embryonic survival, altering Hand1 phosphoregulation, and consequently Hand1 dimerization affinities, in NCCs results in severe mid-facial clefting and neonatal death. Hand1 phosphorylation mutants exhibit a non-cell-autonomous increase in pharyngeal arch cell death accompanied by alterations in Fgf8 and Shh pathway expression. Together, our data indicate that the extreme distal pharyngeal arch expression domain of Hand1 defines a novel bHLH-dependent activity, and that disruption of established Hand1 dimer phosphoregulation within this domain disrupts normal craniofacial patterning.

KEY WORDS: Hand1, bHLH, Craniofacial development, Transcription, Dimerization, Phosphorylation, Neural crest

INTRODUCTION

Neural crest cells (NCCs) are a multipotent cell population that specifies within the dorsal lip of the neural tube and subsequently delaminates, migrates and populates the pharyngeal arches (PAs), before ultimately differentiating into a wide spectrum of structures/tissues along the anterior-posterior (AP) axis of vertebrate embryos (Clouthier et al., 2010; Minoux and Rijli, 2010; Ruest and Clouthier, 2009; Trainor, 2005; Gitton et al., 2010). The NCCs that migrate into the first and second PAs are primarily responsible for orchestrating craniofacial development. Well-integrated signaling programs function through transcription factors to define tissue patterning and NCC differentiation. The complex signals that govern craniofacial morphogenesis involve a number of input pathways, including Fgf, Shh, Wnt, Bmp, Pdgf, retinoic acid (RA) and endothelin signaling (Abe et al., 2008; Abzhanov and Tabin, 2004; Clouthier et al., 2003; Jiang et al., 2006; Kurihara et al., 1995; Macatee et al., 2003). Dysregulation of NCC migration, proliferation and patterning can result in craniofacial abnormalities observed in numerous human syndromes (Chai and Maxson, 2006; Jiang et al., 2006; Noden and Trainor, 2005; Clouthier et al., 2013). Indeed, cleft lips and cleft palates are among the most common congenital defects observed in newborns. Understanding the signaling pathways and downstream transcription factors that govern craniofacial morphogenesis is crucial in defining the etiology of these common congenital defects.

Members of the basic helix-loop-helix (bHLH) transcription factor superfamily play dominant roles in cell specification, differentiation and tissue patterning throughout embryonic development (Massari and Murre, 2000). bHLH transcription factors affect transcription by forming either a bHLH homo- or heterodimer and by binding DNA via a cis-element termed E-box. Loss-of-function studies in the Twist sub-class of bHLH factors, which include Twist1, Twist2, Hand1 and Hand2 (Barnes and Firulli, 2009), reveal that these factors play key roles in craniofacial morphogenesis. Targeted disruption of Twist1 in NCCs leads to defects in upper and lower jaw development (Bildsoe et al., 2009). NCC-specific deletion of Hand2 results in disruption of lower jaw and tongue development (Barron et al., 2011). These factors appear highly conserved during vertebrate evolution (Barnes and Firulli, 2009) and probably play similar roles in human facial development. Indeed, human mutations in TWIST1 cause Saethre-Chotzen syndrome (SCS). In addition to craniosynostosis, palatal anomalies are commonly observed in SCS patients (Stoler et al., 2009).

Compelling evidence shows that Twist family bHLH factors mediate biological function by dimer choice (Castanon et al., 2001; Firulli et al., 2003, 2005, 2007). Dimer choice is regulated, in part, by a threonine and serine pair that is evolutionarily conserved among all Twist family members. Mimicking Hand1 hypophosphorylation through mutations in residues T107 and S109 enhances homodimer formation, whereas mimicking Hand1 phosphorylation at T107 and S109 enhances formation of E-protein heterodimers (Firulli et al., 2003). Indeed, changes in bHLH dimer choices affect craniofacial development (Connerney et al., 2006). Dysregulation of Twist1 phosphorylation at these threonine and serine residues causes SCS (Cai and Jabs, 2005; Firulli et al., 2005). Dimerization of Twist-family bHLH factors is governed by their co-expression within a cell, their colocalization within the nucleus, their level of relative expression and the phosphorylation of the conserved threonine and serine residues within Helix I of the bHLH domain (Firulli and Conway, 2008; Firulli et al., 2003, 2005, 2007). Overexpression studies alter the stoichiometry of this bHLH pool. Thus, to validate the consequences of dysregulating this post-translational control mechanism within the bHLH dimer pool in vivo, it is essential to manipulate phosphorylation but not expression levels.

By contrast to the dominant roles exhibited by Twist1 and Hand2 in craniofacial development, the related factor Hand1, although expressed within the distal most NCC-derived PA mesenchyme (Clouthier et al., 2000), shows no observable phenotypes in NCC loss-of-function analysis. However, there is a clear gene dosage effect when Hand1 NCC conditional-null mice are placed on a Hand2 heterozygous background (Barbosa et al., 2007). This suggests a crucial Hand gene dosage that, when disrupted, perturbs the bHLH dimer pool within cranial NCCs, resulting in morphogenic defects. To test whether Hand1 phosphorylation-dependent dimer regulation

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Received 30 December 2013; Accepted 29 May 2014
ALTERS THE COMPOSITION OF THE BHLH DIMER POOL, WHICH INFLUENCES CRANIOFACIAL MORPHOGENESIS, WE ENGINEERED TWO HAND1 CONDITIONAL-ACTIVATION KNOCK-IN ALLELES DESIGNATED TO EITHER MIMIC HAND1 HYPOPHOSPHORYLATION BY REPLACING BOTH THREONINE 107 AND SERINE 109 WITH ALANINES (PO4−), OR TO MIMIC HAND1 HYPERPHOSPHORYLATION BY REPLACING THESE RESIDUES WITH ASPARTIC ACIDS (PO4+). ACTIVATION OF THESE PHOSPHO-MUTANT ALLELES WITHIN NCCS RESULTS IN SEVERE MID-FACIAL CLEFTING. MUTANT EMBRYOS DISPLAY ABNORMAL CELL DEATH WITHIN THE DEVELOPING PAs, RESULTING IN REDUCED OUTGROWTH OF THE MAXILLARY PROCESSES AND ABERRANT CRANIOFACIAL DEVELOPMENT. THE MAJORITY OF STRUCTURAL DEFECTS OCCUR WITHIN TISSUES WHERE HAND1 IS NOT EXPRESSED AND WHERE HAND1-LEVELS ARE NOT DETECTED. GENE EXPRESSION ANALYSES SHOW THAT BOTH BIFURBAST GROWTH FACTOR 8 (FGF8) AND SONIC HEDGEHOG (SHH) SIGNALING PATHWAYS ARE AFFECTED. INTERESTINGLY, REMOVING THE WILD-TYPE HAND1 ALLELE ON BOTH THE HAND1PO4− AND HAND1PO4+ BACKGROUNDS RESULTS IN REDUCED LEVELS OF CELL DEATH AND IMPROVED CRANIOFACIAL DEVELOPMENT. TOGETHER, THESE RESULTS SHOW THAT, ALTHOUGH HAND1 IS DISPENSABLE FOR DEVELOPMENT OF CRANIOFACIAL STRUCTURES, A MISREGULATION OF HAND1 DIMER CHOICE WITHIN THE NCC-DERIVED DISTAL PA ECTOMESENCHYME DISRUPTS THE FUNCTION OF OTHER FACTORS REQUIRED FOR CRANIOFACIAL MORPHOGENESIS. THESE FINDINGS SUGGEST THAT HAND1 AND ITS PUTATIVE BHLH DIMER PARTNERS TRANSCRIPTIONALLY CONTROL ONE OR MORE OF THE THEORIZED CAP SIGNALS.

RESULTS

HAND1 PHOSPHO-MUTANT EXPRESSION WITHIN THE PAs RESULTS IN PRONOUNCED MID-FACIAL CLEFTS

TO UNDERSTAND THE ROLE OF HAND1 PHOSPHOREGULATION WITHIN NCCS, WE ENGINEERED TWO TARGETED CONDITIONALLY ACTIVE HAND1 ALLELES, WHEREIN RESIDUES T107 AND S109 WERE REPLACED WITH EITHER ALANINES (PO4−) OR ASPARTIC ACIDS (PO4+; FIG. 1A). BY INCORPORATING A STOP-FLOX CASSETTE CONTAINING A NEOMYCIN RESISTANCE GENE DOWNSTREAM OF THE HAND1 TRANSCRIPTIONAL START SITE, BUT UPSTREAM OF THE HAND1-INITIATING METHIONINE, WE WERE ABLE TO CREATE NULL, CONDITIONAL PHOSPHO-MUTANT HAND1 ALLELES. IN TISSUES WHERE THE CRE RECOMBINAISE AND ENDOGENOUS HAND1 EXPRESSION DIRECTLY OVERLAP, THE STOP-FLOX CASSETTE IS REMOVED, ALLOWING EXPRESSION OF THE HAND1PO4− OR HAND1PO4+ MUTANT ALLELES. HAND1 EXPRESSION WITHIN NCC-DERIVED ECTOMESENCHYME OF THE DISTAL PAs IS FIRST DETECTABLE AT E9.5. EXPRESSION CANNOT BE DETECTED AT E9.0 USING THE SENSITIVE HAND1PO4−/fx ALLELE (FINULLI ET AL., 1998; FIG. 1B-E). HAND1 EXPRESSION INITIATES IN THE MOST POSTERIOR/CAUDAL PORTIONS OF THE DISTAL PA BEFORE EXPANDING ANTERIORLY/ROSTRALLY, BUT NOT LATERNALLY. AT E13.5, HAND1-EXPRESSING CELLS MARK THE MEDIAL TONGUE (T) AND FUSION POINT OF THE TWO MANDIBULAR HALVES (MD; FIG. 1F,G). ALTHOUGH HAND1-LINEAGE ANALYSIS REVEALS MINOR LATERAL SPREADING OF THESE DISTAL CELLS, HAND1 EXPRESSION IS LOST AS THE CELLS MOVE LATERALLY FROM THE MIDLINER. CRANIOMETRIC STRUCTURES OF THE CALVARIA DO NOT EXPRESS HAND1 OR CONTAIN HAND1 LINEAGE CELLS (BARNES ET AL., 2010).

WE TESTED THE EFFICACY OF OUR CONDITIONAL HAND1 PHOSPHO-MUTANT ALLELES BY ACTIVATING MUTANT EXPRESSION IN NCCS USING THE WNT1-CRE ALLELE (DANIELIAN ET AL., 1998). WHOLE-MOUNT IN SITU HYBRIDIZATION (ISH) OF HAND1 DETECTS DISTAL EXPRESSION IN THE FORMING PAs OF CONTROL EMBRYOS (FIG. 1H-J). TO DEMONSTRATE THAT THE CONDITIONAL ALLELES SHOW THE EXPECTED SPATIAL-TEMPORAL PATTERN, WE CROSSED BOTH THE HAND1PO4− AND HAND1PO4+ MUTANT ALLELES ONTO THE HAND1 CONDITIONAL (HAND1fx) ALLELE (BARBOSA ET AL., 2007; MCFADDEN ET AL., 2005), AND THEN RECOMBINED BOTH CONDITIONAL ALLELES IN NCCS USING THE WNT1-CRE ALLELE. USING HAND1PO4−/fx;Wnt1-Cre(+) AND HAND1PO4+/fx;Wnt1-Cre(+) EMBRYOS, WE DEMONSTRATE THAT mRNA EXPRESSION FROM EITHER OF THE HAND1 PHOSPHO-MUTANT ALLELES IS INDISTINGUISHABLE FROM WILD-TYPE HAND1 OR THE HAND1PO4− EXPRESSION BY HAND1 ISH (FIG. 1J), HOWEVER, WNT1-CRE-MEDIATED EXPRESSION OF BOTH HAND1 PHOSPHO-MUTANT ALLELES RESULTS IN NEONATAL DEATH ACCOMPANIED BY 100% PENETRANT MID-FACE CLEFTS (FIG. 1L-N).

TO DETERMINE WHEN WE COULD FIRST DETECT A FACIAL PHENOTYPE, WE COLLECTED MUTANT EMBRYOS FROM TIMED PREGNANCIES BETWEEN E10.5 AND E19.5. HAND1PO4−;Wnt1-Cre(+), HAND1PO4−/fx;Wnt1-Cre(+) AND HAND1PO4+/fx;Wnt1-Cre(+) PHENOTYPES ARE FIRST MORPHOLOGICALLY IDENTIFIABLE AT E10.5, WHEREIN DISTANCES BETWEEN THE LATERAL NASAL PROMINENCES (LNp) AND THE OLFACTORY PITS (OP, BLACK LINE) ARE EXTENDED AND MAXILLARY PROCESSES (MP) ARE SYMMETRICALLY REDUCED IN SIZE (SUPPLEMENTARY MATERIAL FIG. S1). AS DEVELOPMENT PROCEEDS, THE EXTENT OF THIS PHENOTYPE BECOMES MORE EVIDENT. SURPRISINGLY, REMOVAL OF THE WILD-TYPE HAND1 ALLELE REDUCES THE SEVERITY OF BOTH HYPOPHOSPHORYLATED AND PHOSPHORYLATION-MIMICKING PHENOTYPES. HISTOLOGICAL ANALYSIS AT E14.5 SHOWS THAT STRUCTURES SUCH AS TOOTH PRIMORDIA (TP), MECKEL’S CARTILAGE (MC) AND NASAL CAVITIES (NC) FORM NORMALLY IN THE HAND1 PHOSPHO-MUTANT EMBRYOS (SUPPLEMENTARY MATERIAL FIG. S2). THE NASAL CAPSULE IS ALSO PRESENT BUT IS DEVATED DOWN THE MIDLINER WITH THE REST OF THE FACIAL STRUCTURES. BY CONTRAST, HAND1 PHOSPHO-MUTANT HETEROZYGOTES [HAND1PO4−;WNT1-CRE(+)] AND HAND1PO4+/fx;WNT1-CRE(+) LACK A PATENT NASAL SEPTUM (DOES NOT FUSE AT THE MIDLINER; NS; BLACK ASTERISK; SUPPLEMENTARY MATERIAL FIG. S2G, I). FURTHERMORE, THE PALATAL SHELVES (PS) ARE NOT FUSED, RESULTING IN ABERRANT COMMUNICATION BETWEEN THE NASOPHARYNX AND ORAL CAVITY (SUPPLEMENTARY MATERIAL FIG. S2B, D). SINGLE-COPY POINT MUTANTS [HAND1PO4−/fx;WNT1-CRE(+)] AND HAND1PO4+/fx;WNT1-CRE(+) SHOW IMPROVEMENT IN NASAL SEPNUM DEVELOPMENT (SUPPLEMENTARY MATERIAL FIG. S2H, J), AND IN THE CASE OF HAND1PO4−/fx;WNT1-CRE(+) EMBRYOS, PALATAL SHELF FUSION IS OBSERVED IN MIXED PENETRANCE, ALTHOUGH CLEFTING IS STILL FULLY PENETRANT (SUPPLEMENTARY MATERIAL FIG. S2E, J).

WE NEXT EMPLOYED MICRO-COMPUTED TOMOGRAPHY (MICRO-CT) SCANS OF HEADS FROM P0 HAND1PO4−/fx;WNT1-CRE(+), HAND1PO4−/fx;WNT1-CRE(+) AND HAND1PO4+/fx;WNT1-CRE(+) EMBRYOS. IN HAND1PO4−/fx MUTANT EMBRYOS, STRUCTURAL DEFECTS WERE OBSERVED THROUGHOUT THE SKULL. ON LATERAL VIEW, THE PREMAXILLA (PM, DARK PURPLE) IS SHORTEO, AND BOTH THE OVERLYING NASAL BONE (N, GREEN) AND A PORTION OF THE MAXILLA (MX, BLUE) CLOSEST TO THE FRONTAL BONE (F, RED) ARE MISSING (FIG. 1L). THE JUGAL BONE (J, DARK RED), THE MIDDLE BONE OF THE ZYGOMATIC ARCH (WITH THE OTHERS BEING THE ZYGOMATIC PROCESSES OF THE MAXILLA AND SQUAMOSAL BONES), IS HYPOPLASTIC, AND THE SQUAMOSAL BONE (SQ, LIGHT PURPLE) IS ABSENT (FIG. 1K). THE PROXIMAL MANDBLE (MD, BRONZE) IS ALSO HYPOPLASTIC. IN DORSAL VIEW (FIG. 1L), THE INTERPARietal (I, TURQUOISE) AND Frontal bones are hypoplastic, the latter leading to a large gap between the frontal bones. The Upper incisors (MAGENTA, WHITE ARROW) ARE READILY VISIBLE DUE TO THIS HYPOPLASIA AND THE ABSENCE OF THE NASAL BONE. BOTH SAGITTAL SUTURES ARE ALSO ABERRANTLY FUSED (ASTERISK). THE HYPOPLASIA OF THE MANDBLE IS MORE OBVIOUS FROM THE DORSAL VIEW, WITH THE TWO HALVES FAILING TO MEET AT THE MIDLINER. A VENTRAL VIEW (FIG. 1M) SHOWS LESS SIGNIFICANT CHANGES TO THE SKULL BASE. WHEREAS THE MIDLINER DEFECTS AROUND THE PREMAXILLA ARE OBVIOUS, MOST BONES APPEAR HYPOPLASTIC. THE BASSISPHENOID (BS, UNCOLORED) AND PTERYGOID BONES (PT, UNCOLORED) ARE SEVERELY UNDERDEVELOPED, WITH THE MISSING SQAMOSAL BONE MORE OBVIOUS. ALSO MISSING IS THE LAMINA OBtURANS (THE BONY PORTION OF THE FUTURE ALISPHENOID THAT ABUTS THE SQAMOSAL BONE). IN ADDITION, THE MIDLINER CLEFT DEFECT RESULTED IN THE FAILURE OF THE PALATINE BONES AND THE PALATAL PROCESSES OF THE MAXILLA TO FUSE (PL, YELLOW). TO EXAMINE CARTILAGE DERIVATIVES, WE STAINED E17.0 CONTROL AND HAND1PO4−/fx;
Fig. 1. Expression and craniofacial phenotypes of Hand1 phospho-mutant mice. (A) Targeting design and Southern blot restriction fragment length polymorphism (RFLP) analysis of the Hand1PO4− and Hand1PO4+ mutant alleles. (B-G) β-galactosidase staining of Hand1lacZ embryos (hearts removed prior to staining) at E9.0 (B,C), E9.5 (D,E) and E13.5 (F,G) showing the distal expression of Hand1 initiating within the first arch (marked in B,C as ‘I’) between E9.0 and E9.5 (black arrow in E). Expression is limited to the most distal arch tissue, which at E13.5 marks the central tongue (t) and mandible (md). (H-J) Whole-mount ISH showing Hand1 expression within the medical arch mesenchyme in wild type (H) and in Hand1PO4−/− (I) and Hand1PO4+/− (J) single copy mutants (hearts removed prior to hybridization). (K-M) Micro-CT images of P0 skulls from control, Hand1+/−PO4−, Hand1PO4−/−, Hand1+/−PO4+ and Hand1PO4+/− embryos shown from the right lateral (K), dorsal (L) and ventral (M) sides of the skulls. Abbreviations for row K: i, interparietal bone (turquoise); p, parietal bone (light green); f, frontal bone (red); n, nasal bone (green); sq, squamosal bone (light purple); pm, premaxilla (dark purple); j, jugal bone (dark red); mx, maxilla (blue); md, mandible (bronze); ty, tympanic bone (uncolored). Abbreviation for row L: i, incisors (magenta, indicated by white arrow). Abbreviations for row M: pl, palatine bones (yellow); bs, basisphenoid (uncolored); pt, pterygoid bones (uncolored). n≥4 for each genotype. Bone and cartilage staining of control (N) and Hand1PO4−/− mutant (O). Maxilla, (mx); hyoid (hy).
**Hand1** phospho-mutants exhibit increased cell death within the PAs

We postulated that the midline defects in facial structures could be the result of a defect in cell migration, the inability of the structures to fuse, decreased cell proliferation or increased cell death within the cranial NCC. We looked at NCC migration by utilizing ROSA26<sup>Rare</sup> reporter lineage-tracing and observed no obvious migration defects for the NCCs entering the PAs or cardiac outflow tract (supplementary material Fig. S3). Defects in tissue fusion seem unlikely, as fusion is observed in the **Hand1**<sup>PO4<sup>/−</sup>;**Wnt1-Cre<sup>/+</sup></sup> mutants (Fig. 1; supplementary material Fig. S2), and the assessment of the fusion markers Jagged2 and Mmp13 reveal no significant variations between E14.5 control and **Hand1**<sup>PO4<sup>/−</sup>;**Wnt1-Cre<sup>/+</sup></sup> or **Hand1**<sup>PO4<sup>/−</sup>;**Wnt1-Cre<sup>/+</sup></sup> embryos (supplementary material Fig. S4). 5-Ethynyl-2′-deoxyuridine (EdU) incorporation analysis reveals no significant difference in NCC proliferation between control and all **Hand1** phospho-mutant embryos at E9.5 and E10.5 (data not shown).

By contrast, significantly elevated levels of cell death are observed within the PAs of **Hand1** phospho-mutant embryos compared with control littermates (Fig. 2). At E9.5, whole-mount LysoTracker staining of control embryos reveals two dorsally localized domains of normal developmental cell death (black arrows) with low levels of cell death detected within the PA mesenchyme (Fig. 2A, white arrow). This is confirmed by section terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) analysis (Fig. 2C, yellow arrows indicating developmental cell death). E9.5 **Hand1**<sup>PO4<sup>/−</sup>;**Wnt1-Cre<sup>/+</sup></sup> mutant embryos exhibit a marked reduction in the developmental dorso-lateral cell death domains while displaying a marked increase in PA cell death as assayed by both LysoTracker and TUNEL staining (Fig. 2E,G).

Correlating with the less severe clefting phenotype, **Hand1**<sup>PO4<sup>/−</sup>;**Wnt1-Cre<sup>/+</sup></sup> mutants display higher levels of normal dorso-lateral developmental cell death similar to controls, while at the same time exhibiting decreased cell death within the PA mesenchyme (Fig. 2L,K).

Similar findings are observed in **Hand1**<sup>PO4<sup>/−</sup>;**Wnt1-Cre<sup>/+</sup></sup> embryos (Fig. 2M,O). At E10.5, the extent of cell death within the PA of the phospho-mutants is reduced when compared with control embryos (Fig. 2B,D,F,H,J,L,N,P,R,T). Given that the **Hand1** expression domain (Fig. 1) does not directly overlap with the observed domain of PA cell death, we reasoned that a cell signaling pathway is disrupted in **Hand1** phospho-mutant embryos and that the observed craniofacial phenotypes are generated non-cell autonomously.

### **Hand1** phospho-mutants show alterations in signaling pathways that regulate craniofacial formation

During craniofacial formation, numerous signaling pathways intersect to govern normal morphological patterning and include Fgf, Shh, Wnt, Bmp and RA signaling (Clouthier et al., 2010; Trainor, 2005).

We first looked at altered RA signaling as **Rdh10** mutant mice exhibit similar mid-facial clefting (Sandell et al., 2007). By intercrossing **Hand1** phospho-mutant mice with the **RARE-lacZ** reporter line (Rossant et al., 1991), we found that **RARE-lacZ** patterning was largely unaffected in both **Hand1**<sup>PO4<sup>/−</sup>;**RARE-lacZ<sup>+/−</sup>;**Wnt1-Cre<sup>/+</sup></sup> and **Hand1**<sup>PO4<sup>/−</sup>;**RARE-lacZ<sup>+/−</sup>;**Wnt1-Cre<sup>/+</sup></sup> embryos at E10.5 and E11.5 (supplementary material Fig. S5). Likewise, expression of both Bmp2 and Bmp4 shows no appreciable difference between mutant and control embryos (data not shown).

We next examined **Fgf8** expression in control and **Hand1** mutant embryos between E9.5 and E12.5 (Fig. 3). Mouse models that exhibit variation in **Fgf8** gene dosage and **Fgf8** gain-of-function studies in chicks both present with mid-face clefts that are accompanied by early cell death (Abzhanov and Tabin, 2004; Chen et al., 2012; Griffin et al., 2013; Moon and Capecchi, 2000). **Fgf8** expression between E9.5 and 12.5 shows a marked expansion in **Hand1** phospho-mutants when compared with control embryos. **Fgf8** expression within the rostral portion of the first arch, the maxillary processes and surrounding the nasal pits is expanded and becomes more pronounced over time (Fig. 3: compare D with H, L, P and T). Levels of **Fgf8** expression appear more persistent in E12.5 mutant embryos where olfactory pit expression is more robust and expression at the edge of the maxilla is still visible (Fig. 3D,H,L,P,T, white arrows).

To validate this observation, we next looked at the expression of sprouty homolog 1 (Spry1) and its variants 5 (**Spry1<sup>5</sup>**), both downstream mediators of **Fgf8**/**Fgfr1** signaling (Firnberg and Neubüser, 2002; Lunn et al., 2007; Minowada et al., 1999; Raible and Brand, 2001), in **Hand1**<sup>PO4<sup>/−</sup>;**Wnt1-Cre<sup>/+</sup></sup> and **Hand1**<sup>PO4<sup>/−</sup>;**Wnt1-Cre<sup>/+</sup></sup> embryos at E10.5 (Fig. 4). Similar to **Fgf8** expression, **Spry1** expression is expanded around the first PA and surrounding the olfactory pits in both **Hand1**<sup>PO4<sup>/−</sup>;**Wnt1-Cre<sup>/+</sup></sup> and **Hand1**<sup>PO4<sup>/−</sup>;**Wnt1-Cre<sup>/+</sup></sup> mutants.
when compared with control littermates (Fig. 4B,C, asterisks). Expression of *Etv5* is also expanded. In control embryos, *Etv5* mRNA is observed to be more robust within the rostral portion of the first PA (Fig. 4D, black arrow). In comparison, *Etv5* expression within the first PA of both the *Hand1*+/PO4− and *Hand1*+/PO4+ phospho-mutant embryos shows a clear caudal expansion (Fig. 4E,F, white arrows). The expression of the *FgfR1* shows no significant differences in expression patterns (Fig. 4G-I). We next performed qRT-PCR on the first PA of both the *Hand1*+/PO4− and *Hand1*+/PO4+ phospho-mutant embryos. We also performed qRT-PCR on the first PA of both the *Hand1*+/PO4− and *Hand1*+/PO4+ phospho-mutant embryos.

**Fig. 2.** Cell death analysis in *Hand1* phospho-mutant embryos. Control (A-D), *Hand1*+/PO4− (E-H), *Hand1*PO4−/fx (I-L), *Hand1*+/PO4+ (M-P) and *Hand1*PO4+/fx (Q-T) were assayed for cell death using LysoTracker (whole mounts) and TUNEL (sections) at E9.5 and E10.5. Black arrows in whole mount and yellow arrows in sections mark developmental cell death observed in all controls, white arrows indicate cell death within the PAs that is increased in *Hand1* phospho-mutants. n≥4 for each genotype.

**Fig. 3.** *Fgf8* craniofacial expression in *Hand1* phospho-mutant embryos. Control (A-D), *Hand1*+/PO4− (E-H), *Hand1*PO4−/fx (I-L), *Hand1*+/PO4+ (M-P) and *Hand1*PO4+/fx (Q-T) were assayed for *Fgf8* expression by whole-mount ISH. Asterisks show enhanced arch expression at E9.5. White bars in B-D,F-H show size differences in the diameter of the olfactory pits, and black bars in C,D,G,H show differences in distance between the olfactory pits of control and *Hand1*+/PO4− mutants. n≥4 for each genotype. White arrows in the bottom row indicate persistent *Fgf8* expression.
Hand1 phospo-mutants exhibit a similar head expansion between the nasal pits (supplementary material Fig. S1). At E9.5, expanded expression of Shh is observed within Hand1 phospho-mutant embryos when compared with controls (Fig. 5A-E, white asterisks). E11.5 control embryos (Fig. 5F) show undetectable Shh expression within the medial head ectoderm of the sinus cavity region, although robust expression is observed in the tooth primordia. By contrast, Shh expression is persistent in the sinus cavity of Hand1 phospho-mutant embryos (Fig. 5G-K, white arrows) in addition to the tooth primordia (tp). Persistent Shh medial head ectoderm expression is observed up to E12.5 (Fig. 5K-O), with removal of the Hand1 wild-type allele reducing the observed persistent expression. Additionally, strong Shh E12.5 expression is observed within the forming tooth primordia, rugae (r) and mystacial vibrissae (mv).

We next looked at the expression of patched 1 (Ptc1) in E9.5 embryos (Fig. 6A-C). When compared with control embryos, Ptc1 expression appears unchanged in the forming head mesenchyme and first arch (black asterisk) of both Hand1+/−/PO4−;Wnt1-Cre(+) and Hand1+/−/PO4−;Wnt1-Cre(+) embryos. Enhanced PA expression (black asterisk) of the Shh-regulated transcription factor Gli1 is observed in Hand1 heterozygous phospho-mutants; however, no significant changes are observed in the expression of Gli3 (Fig. 6D-I). To confirm these observations, we performed qRT-PCR on Hand1+/−/PO4−; Wnt1-Cre(+) and Hand1+/−/PO4−;Wnt1-Cre(+) phospho-mutant head cDNA (Fig. 6J). Results confirm that both Shh and Gli1 expression are significantly enhanced in both Hand1 phospho-mutants; however, Ptc1 and Gli3 expression levels are not significantly affected. Collectively, these data demonstrate non-cell-autonomous changes in both Fgf8 and Shh signaling pathways in Hand1 phospho-mutant embryos.

Expression of Twist1 is altered in Hand1 phospho-mutant embryos

As Twist1 is a known integrator of Fgf and Shh signaling (Hornik et al., 2004), we next investigated the expression of Twist1 in control and Hand1 phospho-mutant embryos (Fig. 7). Twist1 loss-of-function mutations are associated with craniofacial defects during embryogenesis (Chen and Behringer, 1995) and the disease SCS (Barnes and Firulli, 2009; Firulli et al., 2005; Jabs, 2004). At E10.5, Twist1 is robustly expressed within the NCC of the medial and lateral nasal processes (np), the maxillary process (mp), as well as the first (I) and second (II) PAs (Fig. 7A). In E10.5 Hand1 phospho-mutant embryos, the Twist1-expressing tissues are visibly reduced in size, including the maxillary process (Fig. 7B-E, white arrows), the medial and lateral nasal processes (Fig. 7B-E) and the visibly smaller first and second PAs (Fig. 7B-E, asterisks). The frontal view of E11.5 control embryos shows the correct juxtaposition of the medial and lateral nasal processes, whereas the Hand1 phospho-mutant embryos show an obvious reduction of Twist1-expressing mesenchyme (black arrows) within both the lateral and medial nasal processes of Hand1 phospho-mutant embryos (Fig. 7F-J) in the sinus cavity (Fig. 7P-T, asterisks), suggesting a loss of tissue. As expression levels of Twist1 mRNA do not appear uniformly reduced, we employed qRT-PCR analysis to quantitate the Twist1 levels more accurately (Fig. 7U). Results show that indeed the levels of Twist1 are significantly increased in both E10.5 Hand1+/−/PO4− and Hand1+/−/PO4−. Thus, it appears that a loss of tissue is responsible for the altered Twist1 expression pattern and that Twist1 expression in both the remaining NCC- and non-NCC-derived mesenchyme is increased in Hand1 phospho-mutants.
Inhibition of Shh signaling partially rescues Hand1 phospho-mutant mid-face clefting

Given the upregulation of Fgf8, Shh and Twist1, we reasoned that we could improve the mid-face clefting phenotype by reducing the gene dosage of Twist1. To test this, we crossed the Hand1+/PO4−; Wnt1-Cre (+) alleles onto the Twist1+/− allele and looked for improvement of mid-face clefting at E11.5 (Fig. 8A-F). Results show no improvement to mid-face clefting in Hand1+/−; Twist1+/−; Wnt1-Cre (+) embryos, and Hand1+/−; Twist1−/−; Wnt1-Cre (+) superimposes Twist1-mediated exencephaly (Chen and Behringer, 1995; Soo et al., 2002) on the Hand1 phospho-mutant clefting phenotype (Fig. 8E,F). We also reasoned that Hand2 expression levels could influence the Hand1 phospho-mutant phenotypes. We therefore crossed Hand1PO4−; Wnt1-Cre (+) mice onto the Hand2−/− background; however, lowering Hand2 gene dosage had no visible effects on Hand1 phospho-mutant craniofacial phenotypes (Fig. 8G-I).

We next reasoned that inhibition of Shh signaling could potentially improve Hand1 phospho-mutant mid-face clefting, so we injected pregnant dams with cyclopamine (Kasberg et al., 2013; Lipinski et al., 2008) to antagonize Shh pathway activation and harvested embryos at E13.5 and E14.5 (Fig. 8J-U). Partial phenotype rescue was observed in the most severe Hand1−/−; Wnt1-Cre (+) mutant phenotype (three out of 15 mutant embryos isolated), as compared with the 100% penetrance of pronounced mid-face clefting observed in Hand1−/−; Wnt1-Cre (+) mice (Fig. 8J-L). The least severe Hand1+/−; Wnt1-Cre (+) mutants show more efficient rescue (seven out of nine mutants; Fig. 8N,O,T,U). It is established that abnormally low and high levels of Shh can also result in facial abnormalities (Lipinski et al., 2010), and the improved phenotype observed further supports the notion that the increased Shh level observed in Hand1 phospho-mutants contributes directly to the mid-face cleft phenotype.

DISCUSSION

The bHLH transcription factor Hand1 is clearly dispensable for craniofacial formation (Barbosa et al., 2007). However, altering the phosphoregulation of Hand1, which influences its bHLH dimerization, within the distal-most pharyngeal arch mesoderm results in a severe non-cell-autonomous increase in PA NCC death that ultimately manifests in a mid-face cleft phenotype. In addition to increased cell death, essential signaling pathways required for proper craniofacial morphogenesis (Fgf8 and Shh) show expanded expression in Hand1 phospho-mutant embryos. Treatment in utero with the Shh antagonist cyclopamine can improve the severity of the resultant mid-face cleft (Fig. 8), thus supporting the hypothesis that the increases observed in these signaling pathways drive the resultant phenotype.

Hand1 expression is observed only within the most distal arch mesenchyme and becomes detectable between E9.0 and E9.5 (Fig. 1; Firulli et al., 1998; Ruest et al., 2004). As the Hand1PO4−/− and Hand1PO4− mice are knock-in alleles, these Hand1 dimer mutants are expressed at endogenous levels in a spatio-temporal pattern consistent with wild-type Hand1. At E9.5, Hand1 phospho-mutants display robust pathological cell death in the PAs, indicating that the deleterious effects from the expression of the mutant allele are nearly immediate within the arch mesenchyme. Indeed, phenotypic changes are present by E10.5 (supplementary material Fig. S1). Histological analysis indicates that right and left structures are essentially normal, although, due to the loss of tissue from the early cell death, the outgrowth of the right and left sides of the forming face fall to fuse (supplementary material Fig. S2). This conclusion is supported by the normal expression of the fusion markers jagged 2 (Jag2) and matrix metallopeptidase 13 (Mmp13) (supplementary material Fig. S4) and the direct initiation of fusion observed in Hand1PO4−/−; Wnt1-Cre (+) embryos (Fig. 1N; supplementary material Fig. S2), which exhibit the least amount of early cell death and a less severe phenotype. Additionally, by utilizing the P0-Cre mouse strain to activate Hand1 mutant allele expression within the NCC during their migration [a full day later than Wnt1-Cre allelic activation (Yamauchi et al., 1999)], the severity and penetrance of the mid-face cleft is greatly reduced (supplementary material Fig. S6). This reinforces the idea that the deleterious effects of Hand1 phospho-mutant proteins are immediate to their expression and that even a subtle delay in expression largely limits...
the observed phenotype. Combined, these data demonstrate that Hand1 phospho-mutant facial structures are capable of fusion and, considering the lack of significant changes in cell proliferation, suggest that a non-cell-autonomous increase in arch mesenchyme cell death causes these phenotypes. Given that reduced cell death is observed in the distal most arch mesenchyme where the Hand1 dimer mutants are expressed (Fig. 2), it is probable that Hand1 phospho-mutants interfere with other bHLH dimer choices within this limited Hand1 expression domain. Consequently, this causes the non-cell-autonomous cell death, leading directly to the changes in Fgf8 and Shh signaling pathway expression and ultimately resulting in the defects in the upper bones of the skull. Outside the Hand1 expression domain, we observe a loss of Twist1-expressing tissue but an increase in Twist1 expression (Fig. 7), which could lead to increased formation of Twist1 homodimers. Indeed, increased Twist1 homodimer expression is directly associated with premature ossification and suture fusion accompanied by enhanced Fgf signaling (Connerney et al., 2006, 2008). Conversely, later-stage deletion of Twist1 using Tyr-Cre also yields a mid-faced cleft (Bildsoe et al., 2009), reinforcing the notion that altered gene dosage in conjunction with dimer choice can dramatically effect development. Initially, we were surprised by the observation that both the hypophosphorylated and phosphorylation-mimicking Hand1 mutants exhibit a less severe phenotype when the wild-type Hand1 allele is deleted. This observation highlights the mechanics of bHLH factors and their prerequisite homodimer or heterodimer formation conveying transcriptional activity on target genes. It is clear that complete...
deletion of Hand1 does not significantly affect the bHLH dimer pool within the distal mesenchyme, as such Hand1 loss-of-function mutants are phenotypically normal, viable and fertile (Barbosa et al., 2007). The absence of Hand1-mediated defects might be the result of functional compensation by the related bHLH factor Hand2 within the cranial NCC. This is an established mechanism that functions in both sympathetic neurons and the developing heart (Barbosa et al., 2007; Hendershot et al., 2008; Howard, 2005; McFadden et al., 2005; Vincentz et al., 2012). By contrast, altering Hand1 phosphorylation alters the ability of Hand1 to interact with potential bHLH dimer partners (Firulli et al., 2003). By interfering with the function of other crucial bHLH factors via direct dimerization and/or by titration of available E-protein levels, the result is a dramatic increase in PA cell death. The finding that wild-type Hand1, in the presence of the Hand1 phospho-mutant alleles, was bred onto a R26RloxP homozygous background, and females of this genotype were crossed to Wnt1-Cre (provided by Dr Philippe Soriano, Mount Sinai Hospital). Both Hand1-PO4- and Hand1+/PO4+ alleles were assayed for Hand1+/PO4+/fx control, embryos show strong Twist1 expression within the lateral nasal process (lnp) and maxillary process (mp), as well as first (I) and second (II) PAs. (B-E) In Hand1 phospho-mutants, Twist1 expression is visibly reduced within both PAs and the maxillary process (white arrow). Frontal views of E11.5 control (F) and mutant (G-J) embryos show a reduced level of expression within the medial nasal process (mnp) as well as a loss of medial expression between the olfactory pits (demonstrated by white line in G). Ventral views of F-J show Twist1 expression within the head mesenchyme. E12.5 control (P) and Hand1 phospho-mutant heads (Q-T). (U) qRT-PCR analysis of Twist1 expression. Error bars denote s.e.m.; *P<0.05 and **P<0.01 by Student’s t-test. n≥4 for each genotype.

MATERIALS AND METHODS

Mouse strains, genotyping and cyclopamine injections

Hand1stopfloxHand1T107.S109A (Hand1PO4−) and Hand1stopfloxHand1T107.S109D (Hand1PO4+) mice were generated from embryonic stem cells targeted with the constructs described in Fig. 1. Genotyping was performed by Southern blot as described previously (Firulli et al., 1998), or with the allele-specific primers H1 (5′-CTGCCATTGGCTCCGCTAGAGG-3′) and PGK (5′-GGCTGCTAAAGCGCATGCTCCAGACTG-3′), using PCR conditions of 94°C for 1 min, 60°C for 1 min and 72°C for 1 min for 35 cycles. B6.129S4-Gt (ROSA)26Sortm1Sor3 (ROSA26R-β-gal homozygous; R26RloxP) mice were genotyped using a probe located 5′ of the Stop-Flox (provided by Dr Phillippe Soriano, Mount Sinai Hospital). Embryos were flash-frozen for RNA isolation and genotyped from the yolk sac DNA. qRT-PCR was performed on a LightCycler 480II (Roche) using TaqMan primers (Life Technologies) recognizing the following transcripts: Fgf8, Spry1, Fgfr1, Evs5, Evs4, Shh, Ptc1, Gli1, Gli3 and Twist1. heads cut between the first and second PAs from viable embryos were assayed for Hand1+/PO4+ or Hand1PO4+/fx embryos. RARE-lacZ mice [Tg(RARE-Hspa1b/lacZ)12K/J] were obtained from the Jackson Laboratories. P0-Cre mice (Yamauchi et al., 1999) were obtained from Simon J. Conway (Indiana University, USA). Cyclopamine (Sigma) was resuspended at 1 mg/ml in 45% w/v solution in water-2-hydroxpropyl-beta-cyclodextrin solution and administered at a dosage of 20 mg/kg bodyweight twice daily to pregnant dams at E8.5-E10.5 via intraperitoneal injection.

Histology

Embryos (E9.5-E18.5) were fixed in 4% paraformaldehyde, dehydrated, embedded, sectioned and Hematoxylin and Eosin (H&E) stained as described (Firulli et al., 2010). A minimum of four viable embryos per genotype was used for all analyses. All data were collected on a Leica DM5000 B compound fluorescence microscope.

ISH-qRT-PCR

Digoxigenin (DiG)-labeled section and whole-mount ISHs were carried out as described (Barnes et al., 2011; Firulli et al., 2010; Vincentz et al., 2008). qRT-PCR was performed on a LightCycler 480II (Roche) using TaqMan primers (Life Technologies) recognizing the following transcripts: Fgf8, Spry1, Fgfr1, Evs5, Evs4, Shh, Ptc1, Gli1, Gli3 and Twist1. Heads cut between the first and second PAs from viable embryos were flash-frozen for RNA isolation and genotyped from the yolk sac DNA. Total RNA was isolated using a High Pure RNA Tissue Kit (Roche) and cDNA was prepared using a High-Capacity cDNA Reverse Transcription Kit (Life Technologies) following the manufacturer’s protocol. Error bars denote s.e.m. Statistical significance was determined using Student’s t-test. P-values ≤0.05 were regarded as significant and marked in all graphs by a
single asterisk, and *P*-values ≤0.01 are denoted by a double asterisk. n ≥ 4 in all experiments.

**TUNEL, LysoTracker and EdU immunohistochemistry analysis**

TUNEL analysis on sectioned embryos was performed as described (Firulli et al., 2010). LysoTracker (Life Technologies) was incubated with embryos as per the manufacturer’s instructions. Embryos were imaged in a well slide. Cell proliferation was assayed using the Click-IT EdU Imaging Kit (Life Technologies). Pregnant dams were injected with EdU (200 mg/kg body weight) 1 h prior to embryo harvest. Embryos were imaged in a well slide.

**Micro-CT and skeletal preparations**

Craniofacial morphology of P0 mice was assessed using high-resolution desktop X-ray microtomography (micro-CT) SkyScan 1172 imaging system (SkyScan). Skulls were dissected, fixed in 10% neutral buffered formalin and stored in 70% ethanol. Skulls were scanned with an isotropic voxel size of 8 µm, with an energy level of 50 kV and an aluminum 0.5 mm filter. A lower energy source was used to capture regions of undermineralized bone. Two-dimensional (2D) cross-sectional grayscale slices (~600-800 slices per skull) from each skull were reconstructed using NRecon reconstruction software (SkyScan). Reconstructed slices were saved as single grayscale thresholds, with scaling of each image conserved. Images (Medical Imaging Software). All 3D images were created using models using OsiriX version 5.6, imaging processing software for DICOM images (Medical Imaging Software). All 3D images were created using OsiriX version 5.6, imaging processing software for DICOM images. Individual TIFF images and converted to DICOM (Digital Imaging and Communications in Medicine) format. DICOM files were used to create 3D models using OsiriX version 5.6, imaging processing software for DICOM images. All 3D images were created using identical grayscale thresholds, with scaling of each image conserved. Overlaying skeletal structures were removed using a bone removal tool to identify structures of the palate (Fig. 1). n ≥ 4 per genotype scanned and significance (*P*-values ≤ 0.05) determined by Student’s *t*-test. Skeletal preparations were performed as described (Firulli et al., 2005).

Acknowledgements

We thank Danny Carney for technical assistance. Infrastructural support at the Herman B Wells Center for Pediatric Research is in part supported by the generosity of the Riley Children’s Foundation, and the Carrolton Buehl McCulloch Chair of Pediatrics.

Competing interests

The authors declare no competing financial interests.

Author contributions

B.A.F. designed and performed experiments, wrote and edited the manuscript. R.K.F. performed micro-CT analysis. J.W.V. performed data interpretation and consultation in experiment design. D.E.C. performed data interpretation and consultation, wrote and edited the manuscript. A.B.F. designed and performed experiments, performed data interpretation, wrote and edited the manuscript.

Funding

This work was supported by the National Institutes of Health (NIH) [1R0AR061392-03]. Deposited in PMC for release after 12 months.

Supplementary material available online at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.107680/-/DC1

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by FGF signaling and can cause chondrodysplasia when overexpressed. Development 126, 4465-4475.


Supplementary Figure 1: Wholemount views of control (A-F), Hand1\(^{+/PO4-}\) (G-L), Hand1\(^{PO4-/fx}\) (M-R), Hand1\(^{+/PO4+}\) (S-X), and Hand1\(^{PO4+/fx}\) (Y-Zd) embryos between E10.5 and E18.5. At E10.5 visible differences in facial structures are observed between controls (A) and phospho mutants (G, M, S, Y) including measure of the distance between the olfactory pits (op; black lines), size of maxillary process (mp) and frontal, lateral and distal nasal processes (fnp, lnp, mnp) though no significant difference is observed in mandible (md) size. In E11.5 controls (B) the left and right distal nasal process begins to meet at the midline, bringing the olfactory pits closer together. In Hand1 phospho mutants (H, N, T, Z) the distance between the olfactory pits does not decrease. The extent of mid-face clefting is patently obvious at E12.5 (compare C with I, O, U, Za) where the tongue (t) is clearly visible through the cleft. Facial defects progressively become more pronounced at E14.5 (compare D with J, P, V, Zb). Top view of E18.5 controls (E) show the nasal capsule (nc) completely fused whereas phospho mutants maintain unfused nasal capsules (K, Q, W, Zc asterisks). Ventral views reveal a near complete loss of the secondary palate (compare F with L, R, X, Zd; black arrows). \(n\geq4\) for each genotype.
Supplementary Figure 2: Histological analysis of Hand1 phospho mutants at E14.5. Frontal (A-E) and transverse (F-J) sections of Control (A, F) Hand1+/PO4− (B, G), Hand1PO4-/fx (C, H), Hand1+/PO4+ (D, I), and Hand1PO4+/fx (E, J) embryos. Frontal sections show that the tongue (t) fails to drop and that the palatal shelves (ps) fail to fuse (marked by asterisk). Tooth Primordia (tp) meckel’s cartilage (mc) and other structures appear normally formed albeit displaced in location. Transverse sections show that the nasal cavities (nc) form in the phospho mutants; however, the nasal septum is absent from facial structures. n≥4 for each genotype.
**Supplementary Figure 3:** Analysis of NCC migration in Control, \textit{Hand1}^{+/PO4-}, and \textit{Hand1}^{+/PO4+} embryos on the \textit{R26R} background. Lateral views of E9.5 day Control (A), \textit{Hand1}^{+/PO4-} (B), and \textit{Hand1}^{+/PO4+} (C), show no significant changes in the \textit{Wnt1-Cre} activation of β-galactosidase within the NCC. Lateral views at E11.5, robust and indistinguishable numbers of reporter-marked NCC are observed in all of the pharyngeal arches and cardiac outflow tract (oft) of both Control and phos-pho-mutant embryos. (G-I) show transverse sections confirming the presence of β-galactosidase-marked NCC within the OFT. Pharyngeal arches are indicated by roman numerals; left ventricle (lv), atria (a). Somite numbers are indicated in the lower right hand corners of each panel. \textit{n}≥4 for each genotype.
Supplementary Figure 4: E14.5 section in situ hybridization analysis of the fusion markers Mmp13 and Jagged 2 in control (A and D), Hand1+/PO4- (B and E), and Hand1+/PO4+ (C and F) embryos. Control sections show the initiation of fusion of the palatal shelves (ps) just dorsal to the olfactory pits (op). Mmp13 expression marks the surrounding epithelium of the palatal shelves as well as the cells surrounding the nasal septum. In Hand1 phospho-mutant embryos Mmp13 expression patterns are not significantly different. Similarly Jagged2 expression mirrors that of Mmp13 in both control (D) and phospho mutants (E and F). n≥4 for each genotype.
Supplementary Figure 5: β-Galactosidase activity showing expression of the Retinoic Acid Reporter Element (RARE) lacZ reporter in Control (A, D, G), activated Hand1+/PO4- (B, E, H), and Hand1+/PO4+ (C, F, I) backgrounds. E10.5 Phospho mutants show a reduction in frontal nasal process (fnp) tissue; however the RA signaling underlining the tissue is largely unaffected. Similarly at E11.5 no significant difference in RA-induced β- Galactosidase staining is evident in between control (G) Hand1 phospho mutants (H and I). Black arrows point to the forming mid-face cleft. n≥4 for each genotype.
Supplementary Figure 6: Activation of Hand1 phospho-mutant expression using P0-Cre reduces the severity of craniofacial phenotypes. The P0-Cre transgene is expressed in migrating NCCs becoming active a day later than the Wnt1-Cre transgene. (Yamauchi et al., 1999) Comparison of E18.5 day control (A and B) and activated Hand1+/P04- (C and D) heads show that fusion of the nasal capsule (nc) is nearly complete though still not phenotypically normal. Nasal bones (nb) and frontal bones (fb) are reduced in size and the white bar marks the gap between the left and right frontal bones, which is absent in controls. Ventral views (B and D) reveal that palate formation in the Hand1+/P04- embryo is vastly improved. As the P0-Cre transgene is expressed within the pharyngeal arch NCC mesenchyme nearly concurrently with Hand1, the obvious temporal delay of deletion of the Stop-flox cassette supports the data in Figure 2 suggesting that the deleterious effects of Hand1 phospho-mutant expression are immediate upon its initial activation. Trachea (t) is labeled in ventral views. n≥4 for each genotype.
**Supplementary Table 1.** Skeletal lengths and area measurements from Micro-CT scanning.

<table>
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<tr>
<th>Skeletal Region</th>
<th>WT</th>
<th>A+</th>
<th>A-</th>
<th>D+</th>
<th>D-</th>
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<tbody>
<tr>
<td>Mandible Area (mm²)</td>
<td>5.698 ± 0.158*</td>
<td>3.170 ± 0.158*</td>
<td>5.088 ± 0.194*</td>
<td>4.718 ± 0.158*</td>
<td>4.721</td>
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<tr>
<td>Mandible Length (mm)</td>
<td>5.021 ± 0.059*</td>
<td>3.442 ± 0.059*</td>
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<td>4.108 ± 0.059*</td>
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<td>Tympamic Length (mm)</td>
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<td>1.996 ± 0.139*</td>
<td>1.760 ± 0.114*</td>
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<td>Premaxilla Area (mm²)</td>
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<td>0.198 ± 0.037*</td>
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<td>Palate Left Area (mm²)</td>
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<td>0.647 ± 0.050*</td>
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<td>Basisphenoid Area (mm²)</td>
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<td>Presphenoid Area (mm²)</td>
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<td>0.627 ± 0.108</td>
<td>1.478 ± 0.133*</td>
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*Significantly different from WT; ¥significantly different from A+