The Prickle1 gene regulates the differentiation of frontal bone osteoblasts in a new animal model

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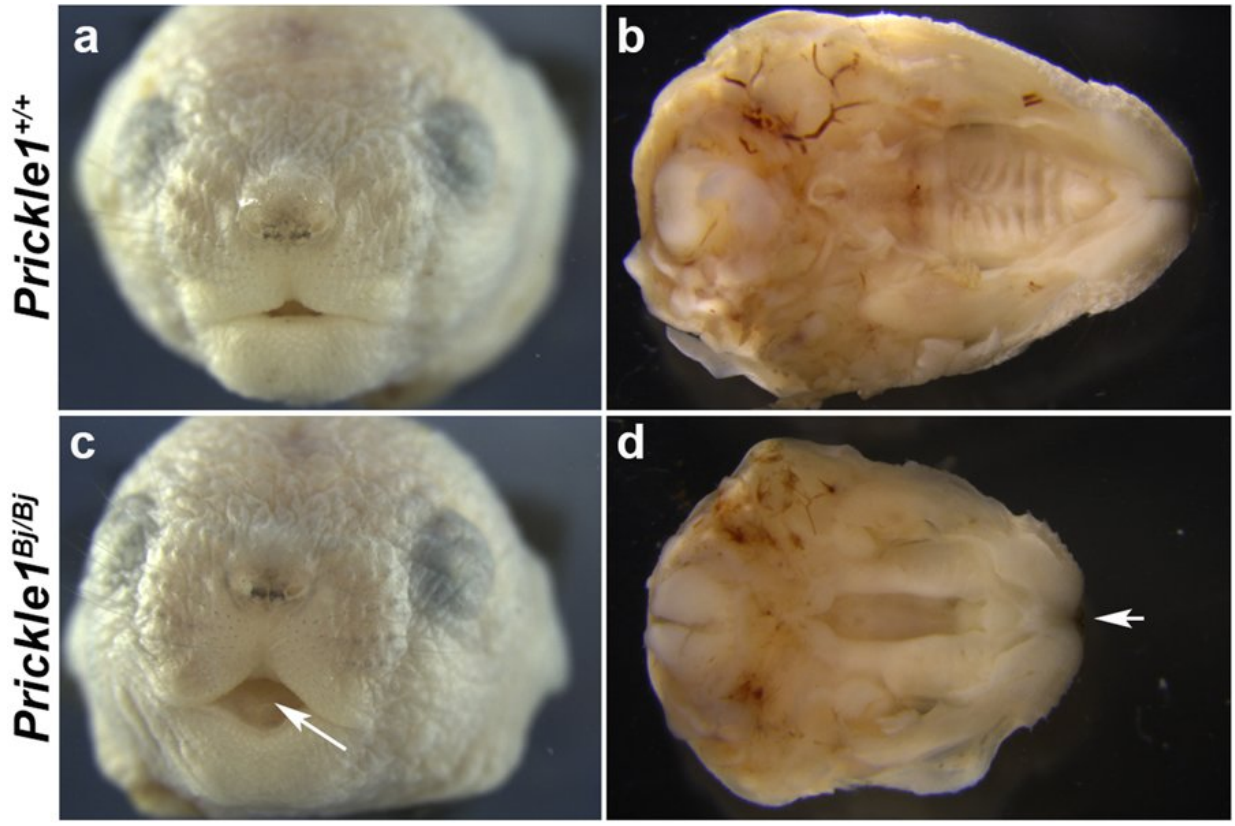


Beetlejuice mutants (Prickle1Bj/Bj) develop median cleft lip and cleft secondary palate. (a,c) Frontal views of wild-type (Prickle1+/+) (a) and Prickle1Bj/Bj (c) at P0 (postnatal day). Median cleft lip indicated by arrow and is completely penetrant. (b,d) Palatal view of fetuses at embryonic stage 18.5 (E18.5). Arrow indicates medial cleft lip and cleft secondary palate. After collection the embryos and P0 fetuses were fixed in 4% paraformaldehyde and embedded in

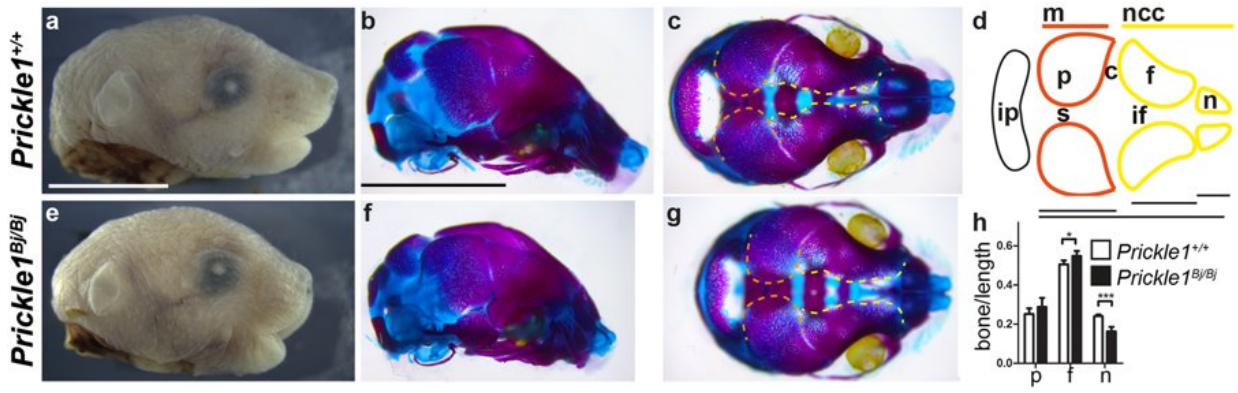
paraffin for histological/immunohistological analysis. Credit: *Scientific Reports*, doi: https://doi.org/10.1038/s41598-018-36742-0

A mechanically compromised skull can result from enlarged fontanelles and smaller frontal bones due to defective migration and differentiation of osteoblasts in the skull primordia (developing skull). The Wnt/Planar cell polarity signaling pathway (Wnt/PCP), usually regulates cell migration and movement in tissues during embryonic development. In a recent study, conducted by Yong Wan and colleagues at the Center for Craniofacial Regeneration, the central research emphasis was on the Prickle1 gene, a core component of the Wnt/PCP pathway, in the skull.

For the studies, Wan et al. used the missense allele of Prickle1, named Prickle1^{Beetlejuice} (Prickle^{BJ}). The homozygous Prickle$^{BJ/BJ}$ 'Beetlejuice' mutants were microcephalic and developed enlarged fontanelles between insufficient frontal bones, although the parietal bones were normal. The homozygous mutants had several other craniofacial defects including a midline cleft lip, incompletely penetrant cleft palate and decreased proximal-distal growth of the head. The scientists observed decreased Wnt/β-catenin and hedgehog signaling in the frontal bone condensations of the homozygous mutants in the study.

The results are now published on *Scientific Reports*. In the homozygous mutants, the frontal bone osteoblast precursors underwent delayed differentiation, alongside decreased expression of migratory markers, resulting in underdeveloped frontal bones. The study showed that the Prickle1 protein function contributed to both migration and differentiation of bone-forming cells (osteoblast precursors) and its absence in the mutant animal model resulted in the defects. The homozygous mutants (Prickle$^{BJ/BJ}$) developed cardiac outflow tract misalignment and cleft palate, contributing to perinatal death of the

mutant mice. Therefore, the observed phenotypic features were from early to late embryonic stages.



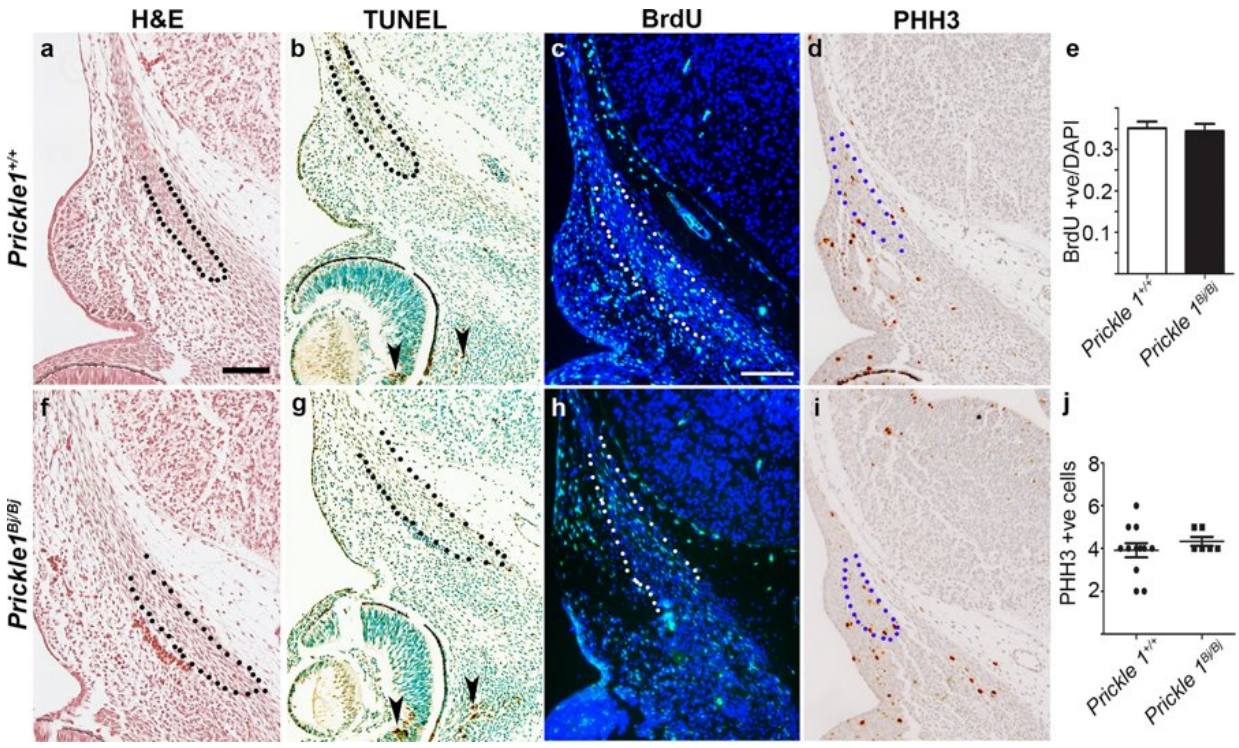
Homozygous mutants (PrickleBJ/BJ) are microcephalic and have defects in the neural-crest derived skull. (a-c, e-g) Macroscopic views of the wild type mouse (Prickle+/+) (a-c) and homozygous mutant (e-g) littermates. d) Schematic of the superior view of the skull vault, h) quantified skull vault measurements. The mutant heads are shorter proximal-distally when observed laterally from the external and with the alizarin red/alcian blue stained specimens. (c,g) Superior view of the skull vault demonstrates the interfrontal suture (yellow lines). d) Schematic of the skull vault showing the tissue origin, below which is the schematic for the measurement in (h). h) The proximal-distal shortening is most profound in the nasal region. Abbreviations: c: coronal suture, f: frontal bone, if: interfrontal suture, ip: interparietal bone, m: mesoderm, n: nasal bone, ncc: neural-crest cell, p: parietal bone, s: sagittal suture. Credit: *Scientific Reports*, doi: https://doi.org/10.1038/s41598-018-36742-0

By nature, the craniofacial complex contains three distinct regions: the skull vault, cranial base and the face. The cranial base forms the floor of the braincase and the skull vault - the roof. Bones of the cranial base form via endochondral ossification, while osteogenesis in the skull vault occurs via intramembranous ossification. Both the skull vault and cranial

base are of embryonic origin (neural-crest derived or mesodermally derived).

In the study model, the Beetlejuice mutants (Bj) contained a point-mutation in the Prickle1 gene (C161F), the Bj C161F mutation was deleterious to the function of the cytoplasmic protein Prickle1. Mutations of the protein in humans are usually associated with familial epilepsy. The mutant phenotype was consistent with another independent point mutation of Prickle1, known as C251X, which included stunted limbs and a cleft palate. While the protein product of the gene is widely expressed in the cytoplasm, little was known about its role in craniofacial osteogenesis.

In the present study, Wan et al. analyzed the bones and cartilage of the head using alcian blue and alizarin red histology dyes. The homozygous mutant skulls were smaller, and the proximal-distal length of the head was reduced with an increased medial-lateral width of the skull. The results showed a statistically significant decrease in the contribution of the nasal bone to the total length of the skull vault in the wild type mice (Prickle$^{+/+}$).

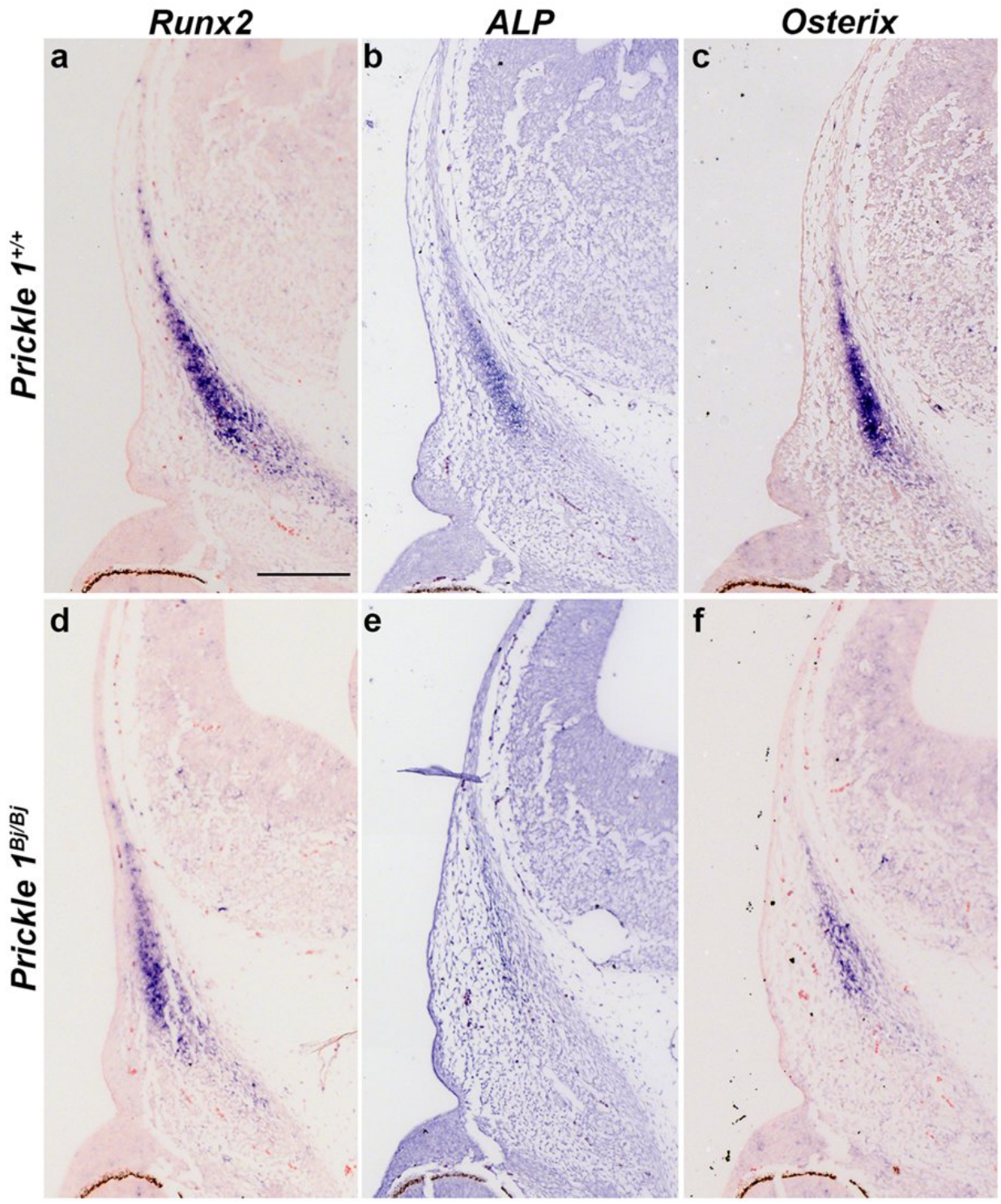


No change in the rate of proliferation or apoptosis in the Prickle1Bj/Bj frontal bones. At embryonic stage 12.5 (E12.5), Prickle1+/+ (a–d) and Prickle1Bj/Bj (f–i) littermates assayed for histology (haemotoxylin and eosin) staining (a,f), TUNEL staining (b,g), proliferation with BrdU immunofluorescence (c,h) and mitosis with phospho-histone H3 immunohistochemistry (d,i). (a,d) The frontal bone mesenchymal condensation (black outline) is present in both wildtype (a) and Prickle1Bj/Bj (f) littermates. (b,g) TUNEL-positive cells were found near the eye (arrowheads) and absent in the frontal bone primordia. (c,h) BrdU-positive cells (green) are found in the frontal bone primordium (white outline) of wildtype (c) and Prickle1Bj/Bj (h) littermates. (d,i) Few positive PHH3-positive cells (brown) are found in the frontal bone primordium of wildtype (d) and Prickle1Bj/Bj (i) littermates. (e) No difference in the ratio of BrdU-positive cells in the frontal bone primordia. (j) No difference in the number of PHH3-positive cells in the frontal bones between genotypes. Credit: *Scientific Reports*, doi: https://doi.org/10.1038/s41598-018-36742-0

In contrast, in the homozygous mutant (Prickle$^{BJ/BJ}$), contributions of the frontal bone to the total length increased, while the proportion of the parietal bone remained unchanged. Taken together, the results indicated that the Prickle1 protein function was required during all stages of frontal bone development.

Wan et al. focused on the function of Prickle1 in the developing skull vault by examining the tissue distribution of the protein in wild type vs. mutant embryos. They found that the Prickle1 mutation resulted in two defected processes during frontal bone development, which included delayed osteoblast differentiation and reduced migration in the frontal bone. Such frontal bone defects were also observed in the phenotypic spectrum of cleidocranial dysplasia (CCD).

The observed frontal bone insufficiency could potentially result from defects in proliferation and cell death. The scientists conducted studies using Haematoxylin and Eosin (H&E) histology dyes to test the hypothesis by observing frontal bone condensation in the wild type vs. mutant animals at embryonic stage 12.5 (E12.5), at which time frontal bone condensation typically occurred. Thereafter, they conducted TUNEL apoptosis assays, where the results indicated very few apoptotic cells (depicted via TUNEL positive uptake) in either genotype.

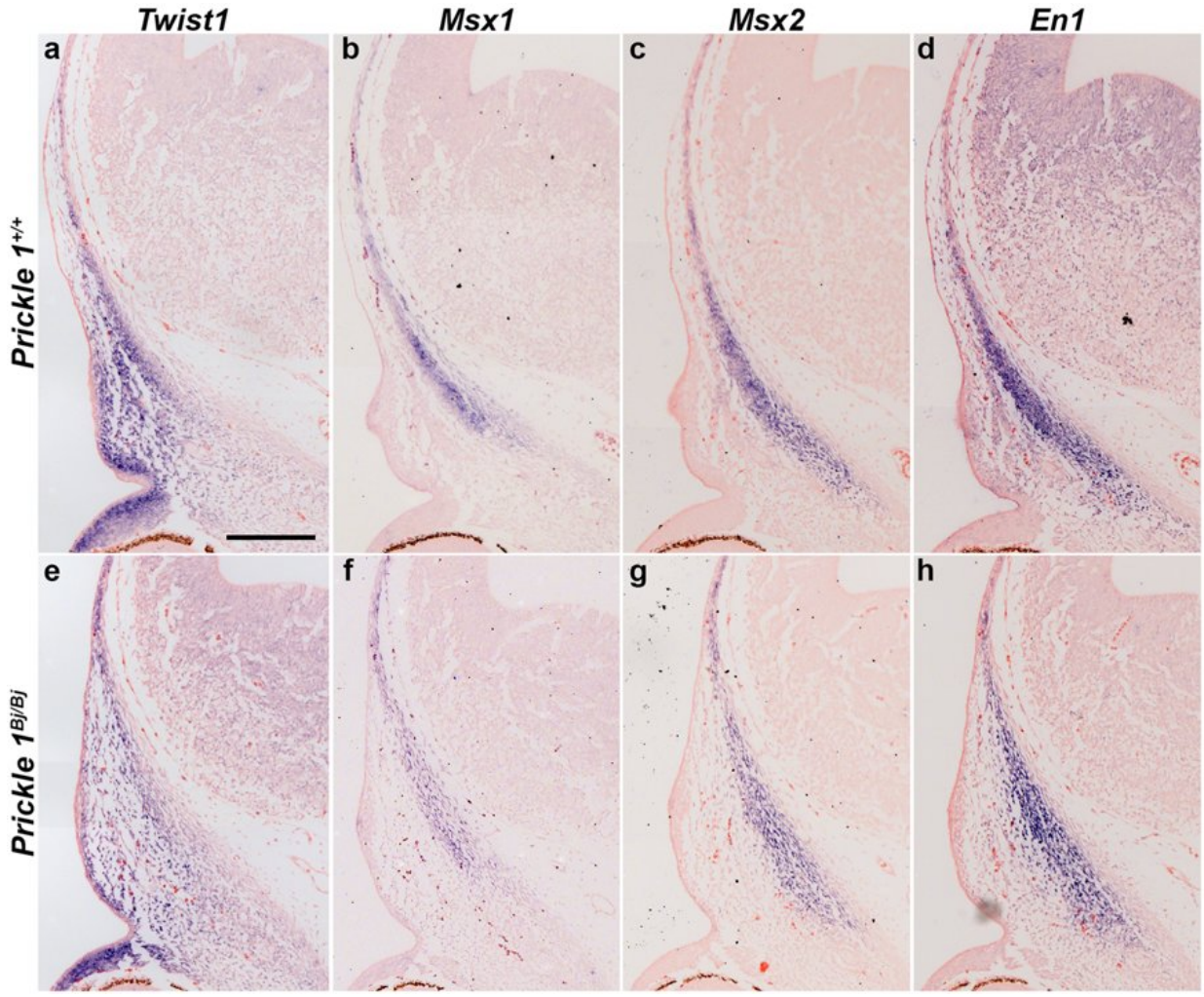


Delayed ossification in the frontal bone primordium. Digoxigenin (DIG)-labeled section in situ hybridization to embryonic stage 12.5 Prickle1+/+ (a–c) and Prickle1Bj/Bj (d–f) littermates. The expression levels of Runx2 (a,d), Alkaline

phosphatase (ALP) (b,e) and Osterix (c,f) are decreased in frontal bone primordium of Prickle1Bj/Bj mutants compared with wild-type control embryos. Credit: *Scientific Reports*, doi: https://doi.org/10.1038/s41598-018-36742-0

The study included BrdU-labelled cell counts in the mutant vs. wild type mice to show no difference in the ratio of proliferating cells either. The number of actively dividing cells were then tested using phosphor-histone H3 immunohistochemistry to show no difference in the number of dividing cells in littermates. Since there was no change in cell death and proliferation, the scientists were next determined to test if osteogenic differentiation was occurring correctly.

For this, Wan et al conducted RNA in situ hybridization experiments to assess the expression of alkaline phosphatase (ALP) and Osterix (OSX, also known as Sp7) in the pre-osteoblasts and osteoblasts of the frontal bones. They determined the expression of RUNX2, an early marker of osteoblast commitment in the skull and of ALP, a marker of more mature osteoblasts. By embryonic stage 15.5 (E15.5), the expression of Runx2, ALP and OSX decreased in the ectocranial layer of the mutant frontal bones compared with wild type littermates. The scientists determined that intramembranous ossification (conversion of mesenchymal tissue into bone) delayed in the frontal bone results in the hypoplastic Beetlejuice mutants.



Osteoblast migration is decreased in the frontal bone primordium. DIG-labeled section in situ hybridization to Twist1, Msx1, Msx2 and Engrailed1 (En1) to E12.5 Prickle1+/+ (a–d) and Prickle1Bj/Bj (e–h) coronal sections. (a,e) Decreased expression of Twist1 in the Prickle1Bj/Bj compared with wild-type. (b,f) The expression of Msx1 is decreased in the mutant compared with wild-type. (c,g) The expression of Msx2 is slightly decreased in the Prickle1Bj/Bj compared with wild-type. (d,h) The expression of En1 is similar in the Prickle1Bj/Bj compared with wild type. Credit: *Scientific Reports*, doi: https://doi.org/10.1038/s41598-018-36742-0

Wan et al. further determined if a defective signaling system led to the

observed delayed frontal bone osteogenesis by studying the level of canonical Wnt and Hedgehog (HH) signaling in the mutants. The results suggested that the levels of HH signaling (required for cranial bone development) were, indeed, defective in the mutant animals.

Finally, they conducted in situ hybridization to markers of osteoblast migration (with markers Engrailed1 (En1), Twist1, Msx1 and Msx2) in the wild type and mutant littermates. The expression level of the markers was reduced in the frontal bone primordia in the mutants. The results suggested the Prickle1 protein function was necessary to mediate cell migration of osteoblast precursors during all stages of skull vault development.

In this way, Wan et al. analyzed the Beetlejuice mutant mouse as a new model to understand the etiology of microcephaly. The number of animal models currently in use to determine the growth patterns of the face and skull in microcephaly are limited. The scientists combined genetic, molecular and physical mechanisms in the study relative to Prickle1 mutants to show contributions to decreased growth of the craniofacial region in the new mouse model. Wan et al. will continue the work to understand how cell migration and the alteration of each compartment (brain, skull vault and cranial base) contribute to the development of microcephaly, clavarial patterning and growth.

More information: Yong Wan et al. Prickle1 regulates differentiation of frontal bone osteoblasts, *Scientific Reports* (2018). DOI: 10.1038/s41598-018-36742-0

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