

## EDITOR'S CHOICE

Animal models are essential tools in the study of orofacial clefting. Our understanding of the molecular genetic mechanisms of clefting has been informed to a great extent by such models. In this study, the authors have developed a new animal model of cleft palate in mice using the technique of transgene insertion mutagenesis. This new animal model will allow the study of various signal transduction pathways that are known to be involved in development of the secondary palate from a new point in those pathways. As such, this model has the potential to significantly advance our understanding of the etiology of clefting of the secondary palate.

## Disruption of the Murine *Ap2 $\beta$ 1* Gene Causes Nonsyndromic Cleft Palate

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**Development of the secondary palate in mammals is a complex process that can be easily perturbed, leading to the common and distressing birth defect cleft palate. Animal models are particularly useful tools for dissecting underlying genetic components of cleft palate. We describe a new cleft palate model resulting from a transgene insertion mutation. Transgene insertional mutagenesis disrupts the genomic organization and expression of the *Ap2 $\beta$ 1* gene located on chromosome 11. This gene encodes the  $\beta$ 2-adaptin subunit of the heterotetrameric adaptor protein 2 complex involved in clathrin-dependent endocytosis. Homozygous cleft palate mutant mice express no *Ap2 $\beta$ 1* messenger RNA or  $\beta$ 2-adaptin protein and die during the perinatal period. Heterozygous mice are phenotypically normal despite expressing diminished  $\beta$ 2-adaptin messenger RNA and protein compared with wildtype. Remarkably, the paralogous  $\beta$ 1-adaptin subunit of the adaptor protein 1 complex partially substitutes for the missing  $\beta$ 2-adaptin in embryonic fibroblasts from homozygous mutant mice, resulting in assembly of reduced levels of an adaptor protein 2 complex bearing  $\beta$ 1-adaptin. This variant adaptor protein 2 complex is, therefore, apparently capable of maintaining viability of the homozygous mutant embryos until birth but insufficient to support palatogenesis. Nonsyndromic cleft palate in an animal model is associated with disruption of the *Ap2 $\beta$ 1* gene.**

KEY WORDS:  *$\beta$ 2-adaptin, clathrin-coated pits, cleft palate, mouse, transgene insertional mutagenesis*

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During morphogenesis of the secondary palate (palatogenesis), bilateral extensions of the maxillary processes (palatal shelves) reorient from a vertical position to a horizontal position over the tongue. Palatal fusion

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Drs. Li and Puertollano contributed equally to this study.

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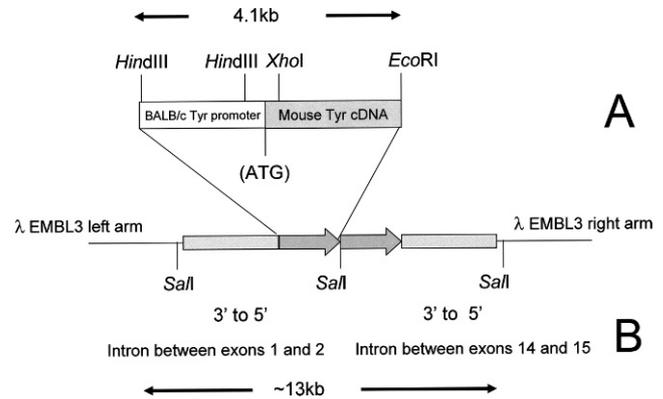
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occurs when there is transformation of the medial edge epithelia (MEE) along with remodeling of the extracellular matrix. Perturbation of this complex cascade of events can lead to cleft palate (CP) (Ferguson, 1988). Nonsyndromic CP (i.e., no other recognizable defects or disabilities) represents a common clinical outcome of genetically heterogeneous etiologies. A growing number of genes that encode transcription factors, signaling molecules, growth factors, growth factor receptors, and extracellular matrix proteins have been implicated in the pathogenesis of syndromic and nonsyndromic orofacial clefts (Schutte and Murray, 1999; Chong et al., 2002; Lidral et al., 2008; Marazita et al., 2009; Shi et al., 2009). Transgene insertional mutagenesis is a common occurrence during the generation of transgenic mice (Meisler, 1992) and has led to the identification of a number of developmentally important genes (Shawlot et al., 1989; Bishop et al., 2000; Overbeek et al., 2001; Cunningham et al., 2002).

Adaptor protein (AP) complexes are composed of subunits (adaptins) that are involved in the formation of intracellular transport vesicles and in the selection of cargo for incorporation into vesicles (Boehm and Bonifacino, 2001). Several heterotetrameric adaptor complexes (AP-1, AP-2, AP-3, and AP-4) are associated with vesicles. Adaptor complexes contain adaptin proteins ( $\alpha$ ,  $\gamma$ ,  $\delta$ , or  $\epsilon$  and  $\beta$ 1,  $\beta$ 2, or  $\beta$ 4, respectively) that are linked to a medium chain ( $\mu$ 1,  $\mu$ 2,  $\mu$ 3, or  $\mu$ 4) and one small chain ( $\sigma$ 1,  $\sigma$ 2,  $\sigma$ 3, or  $\sigma$ 4). The AP-2 adaptor complex ( $\alpha$ ,  $\beta$ 2,  $\mu$ 2, and  $\sigma$ 2) is key to successful clathrin-dependent endocytosis from the plasma membrane; whereas, the AP-1 adaptor complex ( $\gamma$ ,  $\beta$ 1,  $\mu$ 1, and  $\sigma$ 1) is associated with the *trans*-Golgi network and endosomes (Traub, 1997, 2003; Robinson, 2004). The *Ap2β1* gene located on mouse chromosome 11 encodes the AP-2 complex subunit beta-1 protein (AP2B1, UniProtKB/Swiss-Prot Q9DBG3). AP2B1 has been widely studied under several alternative names including adaptor-related protein complex 2 beta-1 subunit,  $\beta$ 2-adaptin,  $\beta$ -adaptin, plasma membrane adaptor HA2/AP2 adaptin beta subunit, clathrin assembly protein complex 2 beta large chain, and AP105B. The *Ap1β1* gene encodes the AP-1 complex subunit beta-1 (AP1B1, UniProtKB/Swiss-Prot O35643). Common alternative names for AP1B1 include adaptor-related protein complex 1 subunit beta-1, adaptor protein complex AP-1 subunit beta-1,  $\beta$ -prime adaptin 1,  $\beta$ 1-adaptin, Golgi adaptor HA1/AP1 adaptin beta subunit, clathrin assembly protein complex 1 beta large chain, and AP105A. To minimize confusion the common alternate names for the products of the *Ap2β1* and *Ap1β1* genes,  $\beta$ 2-adaptin and  $\beta$ 1-adaptin, respectively, are used through this present study.

Herein we report the use of transgene insertional mutagenesis to show that disruption of the *Ap2β1* gene encoding of the  $\beta$ 2-adaptin subunit of the AP-2 complex causes perinatal mortality and nonsyndromic CP.



**FIGURE 1** Schematic representation of the TYBS transgene and genomic clone. **A, B:** The 4.1-kbp TYBS minigene is present as two copies (shaded arrows) in the ~13-kbp  $\lambda$  EMBL3 genomic clone. Genomic DNA flanking the transgene complex corresponds to intronic sequences between exons 14 and 15 and exons 1 and 2 of the *Ap2β1* gene.

## METHODS

### Generation of Transgenic Mice

Transgenic mice were generated by microinjection of a tyrosinase minigene (TYBS) into single-cell mouse embryos (Fig. 1A) (Yokoyama et al., 1990; Overbeek et al., 1991). Albino FVB/NJ female mice mated to FVB/NJ males were used as embryo donors. Outbred ICR females bred to vasectomized (C57BL/6J  $\times$  DBA/2J)F1 males were used as embryo recipients and surrogate mothers. All the animal experiments were performed with the approval of the Indiana University School of Dentistry and Baylor College of Medicine Animal Care and Use Committees.

### Genotyping of Transgenic Mice

The identification of transgenic mice was made initially by the presence of coat color and pigmented eyes. The BALB/c promoter driving the tyrosinase complementary DNA (cDNA) in the tyrosinase minigene provides rescue of the albino phenotype of FVB/NJ mice (Yokoyama et al., 1990; Overbeek et al., 1991). The presence of the transgene in genomic DNA also was confirmed by standard polymerase chain reaction (PCR) using GeneAmp<sup>®</sup> PCR reagents (Applied Biosystems, Foster City, CA) and TYBS-specific primers (TYBS 636F ctgaaatgaggaggacattgatt and TYBS1034R tcaactcagacaaaattccacatt) to amplify a 400-base pair (bp) portion of the TYBS transgene. These primers do not amplify the endogenous *Tyr* gene located on chromosome 7. Tg/+ and Tg/Tg embryos were distinguished using TaqMan<sup>®</sup> Gene Expression Assays (Applied Biosystems). Total cellular RNA was prepared from whole embryos using the RNAqueous-4PCR kit (Ambion, Austin, TX) and reverse transcribed to cDNA, High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) followed by TaqMan<sup>®</sup> Gene Expression

Assays using TaqMan® Universal PCR Master mix (Applied Biosystems), Ap2b1 (Mm00551136\_m1), and Pkg (Mm00435617\_m1), as an endogenous control.

### Genomic Library Construction and Screening

Genomic DNA was prepared from a pool of three homozygous OVE427 CP embryos using the Pure™ Tissue DNA Isolation Systems (DNA Technologies, Inc., Gaithersburg, MD). Following partial digestion with *Sau3AI*, the DNA was size-fractionated using sucrose gradients into two size ranges (9.0 to 13.0 thousand base pairs [kbp] and 13.0 to 23.0 kbp) and then ligated to *BamHI* digested EMBL3 vectors, packaged using Gigapack III packaging extract, and plated on the XL1-Blue MRA (P2) strain (Stratagene, La Jolla, CA). Libraries contained ~5 to 8 × 10<sup>6</sup> clones and unamplified titers of ~1 × 10<sup>10</sup> plaque-forming unit (pfu)/mL. Aliquots of the unamplified 9.0-to-13.0-kbp and 13.0-to-23.0-kbp libraries were screened using PCR (primers described above). The PCR-positive OVE427 library containing 13.0-to-23.0-kbp inserts was plated and the plaque lysates screened by PCR. PCR-positive plates were then subjected to a minimum of three rounds of plaque purification using nonisotopic DNA-DNA hybridization with a digoxigenin (DIG)-labeled TYBS minigene fragment as a probe (DIG-High Prime DNA labeling) and chemiluminescent detection (Roche Diagnostics Corporation, Indianapolis, IN). Two clones were chosen for subsequent amplification and subcloning in pBluescript (Stratagene).

### Northern Analyses

Total cellular RNA was prepared from OVE427 Tg/Tg, Tg/+, and wildtype (+/+) whole embryos 18 days *post coitum* or adult OVE427 +/+ tissues using Tri-Reagent (Molecular Research Center, Cincinnati, OH). Twenty to 35 mg of RNA was separated using a denaturing formaldehyde-1.5% agarose gel and transferred to a MagnaGraph nylon membrane (M.S.I., Westboro, MA). Filters were prehybridized/hybridized using DIG Easy Hyb (Roche Diagnostics Corporation, Indianapolis, IN) and a DIG-labeled cocktail of *Ap2β1* C-terminal and N-terminal probes subcloned from a full length *Ap2β1* cDNA probe (Open Biosystems, Huntsville, AL), washed at high stringency (0.5× SSC/0.1% wt/vol sodium dodecyl sulfate [SDS] at 62°C), then used for chemiluminescent detection on Fuji Super RX medical x-ray film (Fujifilm Medical Systems, Stamford, CT). The membranes were subsequently stripped and reprobed with a DIG-labeled mouse β-actin probe.

### Mouse Embryonic Fibroblasts

Skin from the dorsum of E17-E18 embryos generated by crossing wildtype (+/+) with OVE427 heterozygotes (Tg/+)

and by crossing Tg/+ by Tg/+ was finely minced and placed in DMEM (Gibco, Grand Island, NY) containing 4.5 g/L D-glucose, L-glutamine, sodium pyruvate, 10% FCS (Sigma-Aldrich, St. Louis, MO), and Pen/Strep (Sigma-Aldrich). Cells were cultured at 37°C/5% CO<sub>2</sub> and passaged by lifting with 0.5 g/L trypsin and 0.2g/L EDTA (Sigma-Aldrich) when cells reached 80% confluence.

### Antibodies

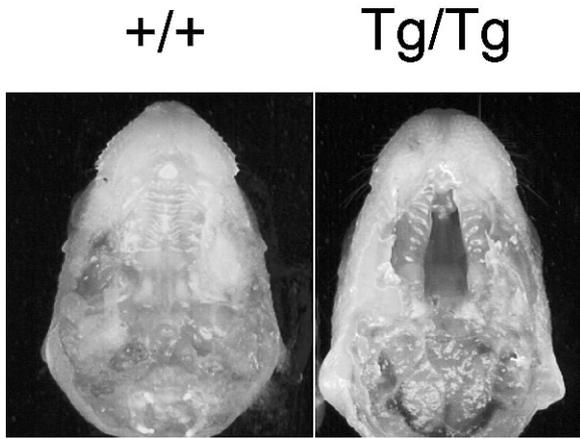
Mouse anti-β2 adaptin, mouse anti-α (A isoform) (Robinson, 1989), and mouse anti-γ (γ1 isoform) monoclonal antibodies (BD Transduction Laboratories, San Jose, CA) were used for immunoblotting and immunoprecipitations in conjunction with horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit IgG (GE Healthcare/Amersham Bioscience, Piscataway, NJ). For immunofluorescence mouse anti-α (Affinity BioReagents, Golden, CO); rabbit anti-epsin, a gift from Dr. Linton Traub (University of Pittsburgh School of Medicine, Pittsburgh, PA); rabbit and mouse antibodies anti-β1+β2 provided by Dr. James Keen (Kimmel Cancer Institute, Philadelphia, PA) and Dr. Tomas Kirchhausen (Harvard Medical School, Boston, MA), respectively, were used in conjunction with goat anti-mouse or goat anti-rabbit IgG conjugated to Alexa Fluor 488 or 555 (Molecular Probes, Eugene, OR).

### Immunofluorescence Microscopy

Wild-type (+/+) and homozygous mutant (Tg/Tg) mouse embryonic fibroblasts (MEFs) were grown on coverslips and fixed in methanol/acetone (1:1, vol/vol) for 10 minutes at -20°C. Incubation with primary antibodies diluted in phosphate buffered saline (PBS), 0.1% wt/vol saponin, and 0.1% wt/vol body surface area (BSA) was carried out for 1 hour at room temperature. Unbound antibodies were removed by rinsing with PBS for 5 minutes, and cells subsequently were incubated with secondary antibody (Alexa555- or Alexa488-conjugated goat anti-rabbit or anti-mouse immunoglobulin) diluted in PBS, 0.1% wt/vol saponin, and 0.1% BSA, for 30 to 60 minutes at room temperature. After a final rinse with PBS, coverslips were mounted onto glass slides with Fluoromount G (Southern Biotechnology Associates, Birmingham, AL). Fluorescence images were acquired on an LSM 510 confocal microscope (Carl Zeiss, Thornwood, NY).

### Immunoblotting and Immunoprecipitation

Fibroblasts were washed with ice-cold PBS, extracted in ice-cold lysis buffer (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.5% vol/vol Triton X-100) supplemented with a protease inhibitor cocktail, Sigma P-8340 (Sigma-Aldrich Co., St. Louis, MO), centrifuged at 16,000 × g for 10 minutes, and supernatants were collected. For



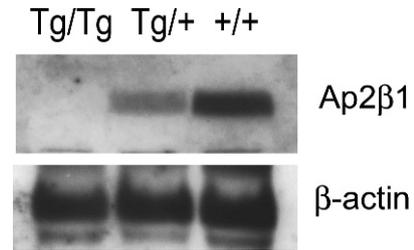
**FIGURE 2** OVE427 wildtype and homozygous embryos. OVE427 Tg/+ male and female mice were mated and embryos 18 days *post coitum* were collected following cesarean section. CP is present in the OVE427 Tg/Tg embryo.

immunoprecipitation, lysates were incubated with 5  $\mu$ L of anti- $\alpha$  (Affinity BioReagents) and protein G-Sepharose (Amersham Bioscience, Piscataway, NJ) at 4°C overnight. Immunoprecipitates were then collected, washed four times with PBS, and eluted by incubation with Laemmli sample buffer (2% wt/vol SDS, 10% vol/vol glycerol, 5% vol/vol 2-mercaptoethanol, 0.002% vol/vol bromophenol blue and 0.0625 M Tris-HCl, pH 6.8) for 10 minutes at room temperature. Samples were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) (4% to 20% gradient gels) under reducing conditions and transferred onto nitrocellulose. The membranes were then blocked with 1 $\times$  PBS, 0.05% vol/vol Tween-20, and 10% wt/vol nonfat milk and incubated with the appropriate antibodies. Enhanced chemiluminescence reagent (Amersham Biosciences) was used for protein detection.

## RESULTS

### Cleft Palate Mutant Strain Developed by Insertional Mutagenesis

The transgenic line OVE427 was found to have perinatal lethality when bred to homozygosity. Complete clefting of the secondary palate was observed in 25% of late gestation embryos produced from intercrossing heterozygous OVE427 Tg/+ mice (Fig. 2). No craniofacial dysmorphology or any anomalies involving the limbs or developing skeleton were noted. Coronal cross-sections of the secondary palate of Tg/Tg embryos at E17 and E18 showed evidence of palatal shelf elevation and the apparent failure of the shelves to fuse. Additional histological examination through serial cross-sections of entire embryos did not reveal differences in major internal organs (heart, brain, lungs, kidneys, and gastrointestinal tract) between wildtype and homozygous littermates other than CP (data not shown).



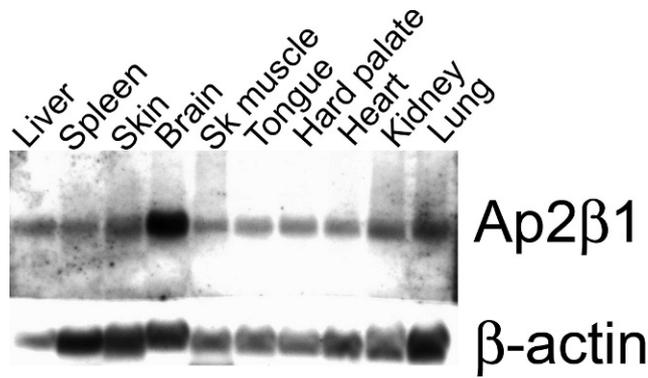
**FIGURE 3** Northern blot analysis of mouse *Ap2β1* and  $\beta$ -actin transcripts in total RNA from OVE427 Tg/Tg, Tg/+, and +/+ embryos. Twenty-five micrograms of total RNA were blotted in each lane. The 5.8-kbp *Ap2β1* transcript is present in heterozygous (Tg/+) and wildtype (+/+) lanes. After hybridization with a cocktail of *Ap2β1* C-terminal and N-terminal probes, the filter was stripped and hybridized with a murine  $\beta$ -actin probe.

### Identification of the *Ap2β1* Gene as the CP Locus

Following *SalI* digestion of a single OVE427 lambda clone, four DNA fragments of approximately 20, 9, 8, and 5 kbp were identified. Southern transfer and hybridization using the TYBS minigene fragment revealed hybridization signals over the 5 and 8 kbp bands and not the 9 and 20 kbp bands (data not shown). The 9 and 20 kbp bands corresponded to the expected sizes of the lambda EMBL3 vector arms. DNA sequencing inward from the EMBL3 vector arms in the undigested lambda clone and of the 5 and 8 kbp *SalI* bands subcloned into pBluescript (Stratagene) was performed. The *SalI* fragments were subjected to secondary digestion using *XbaI*, and those resultant fragments were subcloned and subjected to DNA sequencing. Sequences were analyzed by BLAST® (Basic Local Alignment Search Tool) (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) using the megaBLAST program (compares highly related nucleotide sequences) against all mouse genome assemblies (<http://www.ncbi.nlm.nih.gov/genome/seq/BlastGen/databases.shtml>). From the left arm a 2.3-kbp genomic DNA fragment at 99% identity mapped to chromosome 11 and corresponded to intronic sequence between exons 1 and 2 of the *Ap2β1* (adaptor protein complex 2  $\beta$ 1 subunit) gene (Fig. 1B). From the right arm a 4.2-kbp genomic DNA fragment at 99% identity mapped to chromosome 11 and corresponded to intronic sequence between exons 14 and 15 of the *Ap2β1* gene. These intronic sequences were inverted in orientation in the clone and normally reside ~50 kbp apart in the *Ap2β1* gene. Sequencing within and between the TYBS transgene in this clone confirmed its presence and indicated that two copies of the TYBS transgene integrated in the genome.

### Absence of *Ap2β1* Transcript in Homozygous Mutant Mice

We next investigated *Ap2β1* gene expression by Northern blot. A ~5.8-kbp *Ap2β1* transcript was detected in wildtype (+/+) and heterozygous (Tg/+) embryos but not in RNA prepared from homozygous mutant (Tg/Tg) embryos (Fig. 3). The *Ap2β1* transcript was reduced in intensity in

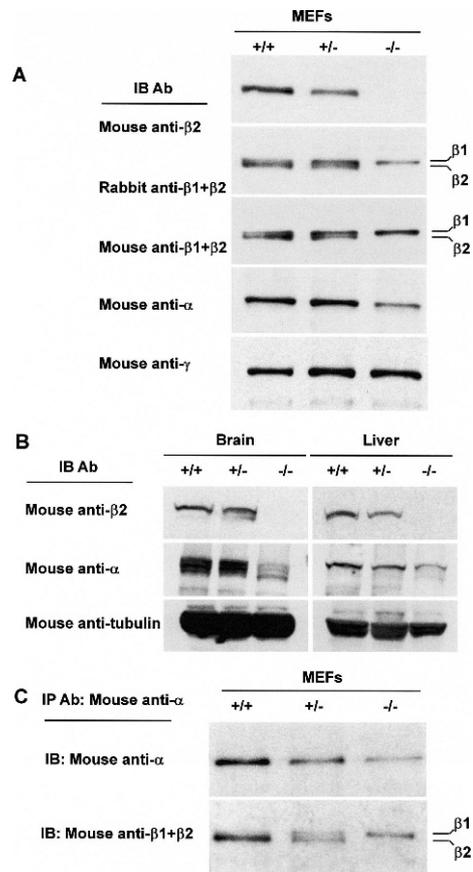


**FIGURE 4** Multi-tissue Northern blot analysis of mouse *Ap2β1* and *β-actin* transcripts in total RNA. Thirty-five micrograms of total RNA were blotted in each lane. The blot was hybridized with a cocktail of *Ap2β1* C-terminal and N-terminal probes, stripped, and rehybridized with a 600-bp murine *β-actin* probe. Tissues surveyed were the liver, spleen, skin, brain, skeletal muscle, tongue, hard palate, heart, kidney, and lung.

the heterozygote when compared with wildtype. A multi-tissue Northern blot showed *Ap2β1* to be widely expressed in wildtype mice, including palatal tissue and with greatest expression in the brain (Fig. 4).

#### Absence of $\beta$ 2-Adaptin Protein in Cells and Tissues From Homozygous Mutant Mice

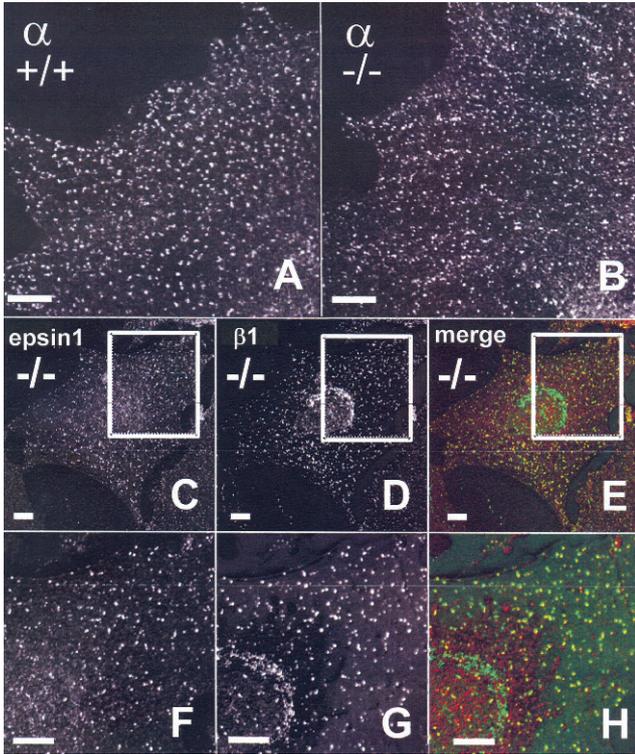
The *Ap2β1* gene encodes a protein named  $\beta$ 2-adaptin that, together with  $\alpha$ -adaptin,  $\mu$ 2, and  $\sigma$ 2, assemble into the heterotetrameric adaptor protein-2 (AP-2) complex involved in clathrin-dependent endocytosis (Boehm and Bonifacino, 2002; Kirchhausen, 2002; Robinson, 2004). Consistent with the Northern analysis, immunoblot analysis with an antibody specific to  $\beta$ 2-adaptin revealed the absence of this protein in mouse embryonic fibroblasts (MEFs) (Fig. 5A) and tissues (i.e., brain and liver) (Fig. 5B) from homozygous mutant (Tg/Tg) animals. MEFs and tissues from heterozygous (Tg/+) mice exhibited reduced levels (~50%) of  $\beta$ 2-adaptin relative to those from wildtype mice. Immunoblot analysis of wildtype MEFs with two different antibodies that recognize both  $\beta$ 2-adaptin and the homologous  $\beta$ 1-adaptin subunit of the AP-1 complex showed a doublet corresponding to the faster-migrating  $\beta$ 2 and the more slowly migrating  $\beta$ 1 (Fig. 5A). MEFs from heterozygous mice exhibited reduced levels of  $\beta$ 2 and unchanged levels of  $\beta$ 1; whereas, those from homozygous mutant mice showed only one expression (Fig. 5A). Levels of  $\alpha$ -adaptin were reduced in homozygous mutant MEFs (Fig. 5A) and tissues (Fig. 5B), suggesting that the absence of  $\beta$ 2-adaptin partially destabilizes  $\alpha$ -adaptin. In contrast, the levels of the  $\gamma$ -adaptin subunit of AP-1 were unchanged (Fig. 5A). These analyses thus showed that homozygous mutant mice did not synthesize any  $\beta$ 2-adaptin and that this deficiency coincided with reduced the levels of  $\alpha$ -adaptin.



**FIGURE 5** Characterization of the AP-2 complex in *Ap2β1* mutant mice. **A, B:** Embryonic fibroblasts or brain and liver tissues were collected from wild-type (+/+), heterozygous (Tg/+), and homozygous mutant (Tg/Tg) mice. After lysis, equal protein loadings of homogenates were analyzed by SDS-PAGE and immunoblotting with the indicated antibodies. **C:** Lysates from (+/+), (Tg/+), and (Tg/Tg) mouse embryonic fibroblasts were immunoprecipitated with mouse monoclonal anti- $\alpha$  as described in the “Materials and Methods” section. Immunoprecipitates were resolved by SDS-PAGE and immunoblotting with antibodies against (upper panel) or  $\beta$ 1+ $\beta$ 2 (lower panel).

#### Partial Substitution of $\beta$ 1-Adaptin for $\beta$ 2-Adaptin in Homozygous Mutant Mice

To examine the assembly status of  $\alpha$ -adaptin in MEFs from wildtype, heterozygous, and homozygous mutant mice, we performed immunoprecipitation of  $\alpha$ -adaptin from cell lysates followed by immunoblotting with an antibody that recognizes both  $\beta$ 1 and  $\beta$ 2 (Fig. 5C). We found that in wildtype MEFs,  $\alpha$  coprecipitated almost exclusively with  $\beta$ 2, as expected for two subunits of the AP-2 complex (Fig. 5C). Strikingly,  $\alpha$  coprecipitated with equal amounts of  $\beta$ 2 and  $\beta$ 1 in heterozygous cells, and only with  $\beta$ 1 in homozygous mutant cells (Fig. 5C). This indicates that  $\beta$ 1 can substitute for  $\beta$ 2 in the AP-2 complex. The amount of  $\beta$ 1 coprecipitated with  $\alpha$  in homozygous mutant cells was higher than that in wildtype cells (in which there is  $\beta$ 2) (Fig. 5C). This means that under normal conditions  $\alpha$  prefers to assemble with  $\beta$ 2 over  $\beta$ 1, but accepts  $\beta$ 1 when there is no  $\beta$ 2.



**FIGURE 6**  $\beta 1$  subunit is partially incorporated into AP-2 complexes in *Ap2β1* mutant mice. A, B: Wild-type (+/+) and homozygous (Tg/Tg) mouse embryonic fibroblasts were grown on coverslips, fixed in methanol:acetone (1:1), and stained with mouse monoclonal antibodies against  $\alpha$  followed by Alexa488-conjugated goat anti-mouse IgG. Cells were examined by confocal fluorescence microscopy. Scale bar, 10  $\mu$ m. C through E: (Tg/Tg) fibroblasts were fixed and double-stained with antibodies against epsin (red) (C) and  $\beta 1$  (green) (D). Bound antibodies were revealed by Alexa-488 conjugated antibody to mouse IgG and Alexa-555-conjugated antibody to rabbit IgG. All images were obtained by confocal microscopy. Merging images in the red and green channels generated the third panel (E and H) on each row; yellow indicates overlapping localization. F, G, and H: Two-fold magnification of the regions shown in panels C, D, and E. Scale bar = 10  $\mu$ m.

### Normal Distribution of the Variant AP-2 Complex Containing $\beta 1$ -Adaptin

We next examined by immunofluorescence microscopy the distribution of the variant AP-2 complex containing  $\beta 1$ -adaptin in wildtype (+/+) and homozygous mutant (Tg/Tg) MEFs. We observed that  $\alpha$ -adaptin localized to punctate foci at the plasma membrane corresponding to clathrin-coated pits in both wildtype and homozygous mutant MEFs (Fig. 6A and 6B). It is interesting that whereas in wildtype cells,  $\beta 1$ -adaptin localizes to the *trans*-Golgi network (TGN) (Robinson, 2004, and data not shown), in homozygous mutant MEFs  $\beta 1$ -adaptin was found on both the TGN and plasma membrane puncta (Fig. 6D and 6G). These puncta, but not the TGN structure, contained another plasma membrane clathrin-associated adaptor, epsin 1 (Fig. 6C and 6F), identifying them as clathrin-coated pits and vesicles.  $\beta 1$ -adaptin (green) and epsin (red) overlap in expression (yellow) (Fig. 6E and 6H). From

these experiments we concluded that incorporation into the AP-2 complex draws  $\beta 1$  to plasma membrane clathrin-coated pits and that the substitution of  $\beta 1$  for  $\beta 2$  in the AP-2 complex has no detectable effect on the localization of this complex.

### DISCUSSION

We have identified a new mouse transgenic insertional mutant that results in autosomal recessive nonsyndromic CP. The transgene integration site is located within the *Ap2β1* gene on chromosome 11, disrupting the genomic organization. Homozygous (Tg/Tg) embryos lack expression of the *Ap2β1* gene as well as the  $\beta 2$ -adaptin protein. Heterozygous (Tg/+) embryos express roughly half the level of wildtype *Ap2β1* transcripts and are completely normal. The *Ap2β1* gene is widely expressed in adult mice, including the hard palate, and is most abundant in brain.

The mechanism by which the loss of  $\beta 2$ -adaptin function leads to CP is not entirely clear. A subunit of the heterotetrameric AP-2 complex,  $\beta 2$ -adaptin is involved in clathrin-dependent endocytosis of receptors from the plasma membrane (Kirchhausen, 2002; Robinson, 2004). It is thus likely that CP in  $\beta 2$ -adaptin-mutant mice results from defective endocytosis of one or more receptors or other cell surface proteins involved in palatogenesis. TGF- $\beta$  superfamily members regulate a wide range of biological processes by binding to two transmembrane serine/threonine kinase receptors (Feng and Derynck, 2005). The TGF- $\beta$ /Smad signaling pathway is known to play a critical role during the process of epithelial-mesenchymal transformation of MEE in palatogenesis (Cui and Shuler, 2000; Tudela et al., 2002; Cui et al., 2003; Cui et al., 2005; Nakajima et al., 2007). Disruption of TGF- $\beta$  signaling can lead to cleft palate (Proetzel et al., 1995; Sanford et al., 1997). Ligand binding leads to both signal transduction and receptor downregulation. TGF- $\beta$  receptor downregulation has been shown to occur through concentration within clathrin-coated pits and internalization into clathrin-coated vesicles for eventual delivery to endosomes (Anders et al., 1997; Anders et al., 1998; Ehrlich et al., 2001; Yao et al., 2002). Clathrin-mediated endocytosis of TGF- $\beta$  receptors depends on the interaction of their cytosolic tails with the  $\beta 2$ -adaptin subunit of AP-2 (Yao et al., 2002). This interaction is in contrast to that of other endocytic receptors, which bind to the  $\mu 2$  subunit (Ohno et al., 1995; Boll et al., 1996; Ohno et al., 1996) or a combination of the  $\alpha$  and  $\sigma 2$  subunits of AP-2 (Chaudhuri et al., 2007; Doray et al., 2007; Mitchell et al., 2008). It is likely that the absence of  $\beta 2$ -adaptin impairs internalization of TGF- $\beta$  receptors from the cell surface, resulting in either sustained signaling from the plasma membrane or decreased signaling from the endosomes (Hayes et al., 2002; Itoh et al., 2002; Di Guglielmo et al., 2003; Runyan et al., 2005). Therefore, the CP phenotype of the  $\beta 2$ -adaptin-deficient mice might result in part from perturbation of TGF- $\beta$  signaling.

Perturbation of  $\gamma$ -aminobutyric acid (GABA)–activated signaling also leads to CP (Homanics et al., 1997; Hagiwara et al., 2003). It is interesting that GABA(A) receptors cycle between the synaptic membrane and intracellular sites; their AP-2-dependent recruitment into clathrin-coated pits represents an important mechanism in the postsynaptic modulation of inhibitory synaptic transmission (Kittler et al., 2000; Herring et al., 2003; Kittler et al., 2005). Therefore, defective GABA(A) receptor internalization and signaling due to the presence of the variant,  $\beta$ 1-adaptin–containing AP-2 complex could also contribute to the CP phenotype of the  $\beta$ 2-adaptin-deficient mice.

In addition to the substitution of  $\beta$ 1-adaptin for  $\beta$ 2-adaptin, the lower levels of the variant AP-2 complex (~50%, as inferred from the levels of  $\alpha$ -adaptin) in the  $\beta$ 2-deficient mice could also be a contributing factor leading to CP. Such substitution of  $\beta$ 1-adaptin for  $\beta$ 2-adaptin has been observed (Keyel et al., 2008). Of note, heterozygous  $\mu$ 2-deficient mice also have reduced levels of the  $\beta$ 2-adaptin AP-2 complex (~70% of  $\alpha$ -adaptin levels seen in wildtype) but display no phenotypic abnormalities (Mitsunari et al., 2005).

Homozygous  $\beta$ 2-adaptin–deficient embryos survive development and die perinatally due to CP. This phenotype contrasts sharply with that of homozygous  $\mu$ 2-deficient embryos, which die before day 3.5 *post coitum* (Mitsunari et al., 2005). As shown here, the reason for the different outcomes likely lies in the unique ability of  $\beta$ 1-adaptin to substitute for  $\beta$ 2-adaptin in the AP-2 complex. Indeed, mouse  $\beta$ 1- and  $\beta$ 2-adaptins are 84% identical in amino acid sequence, a level that to a large extent allows them to behave as interchangeable subunit isoforms (Ahle et al., 1988; Kirchhausen et al., 1989; Ponnambalam et al., 1990; Guilbaud et al., 1997; Boehm and Bonifacino, 2001). This notion is supported by previous work showing that both  $\beta$ 1- and  $\beta$ 2-adaptins promiscuously interact with other subunits from AP-1 and AP-2 in coprecipitation and yeast two-hybrid analyses (Page and Robinson, 1995). An extreme case is *Drosophila melanogaster*, in which a single  $\beta$ 1/2-adaptin is a component of both AP-1 and AP-2 (Camidge and Pearse, 1994). The situation is different for the other three subunits of AP-1 and AP-2, which exhibit 29% to 45% amino acid sequence identity and are not interchangeable (Page and Robinson, 1995). Thus, the inability of  $\mu$ 1 to be incorporated into the AP-2 complex likely accounts for the early embryonic lethality observed with  $\mu$ 2 deficiency (Mitsunari et al., 2005). The converse is also the case because  $\mu$ 2 does not assemble into the AP-1 complex in  $\mu$ 1A-deficient mice, resulting in midgestation embryonic lethality (Meyer et al., 2000). Thus, the survival of  $\beta$ 2-adaptin–deficient embryos until birth likely results from the rescue of most AP-2 functions by substitution with  $\beta$ 1-adaptin.

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