Disruption of the murine beta-adaptin gene leads to non-syndromic cleft palate

Wei Li, Ph.D.[#,*],
Postdoctoral Fellow Department of Oral Facial Development Indiana University School of Dentistry

Rosa Puertollano, Ph.D.[#,*],
Postdoctoral Fellow Cell Biology and Metabolism Program *Eunice Kennedy Shriver* National Institute of Child Health and Human Development (NICHD) National Institutes of Health (NIH)

Juan S. Bonifacino, Ph.D.,
Senior Investigator Cell Biology and Metabolism Program *Eunice Kennedy Shriver* National Institute of Child Health and Human Development (NICHD) National Institutes of Health (NIH)

Paul A. Overbeek, Ph.D., and
Professor Department of Molecular and Cellular Biology Baylor College of Medicine

Eric T. Everett, Ph.D.[*]
Associate Professor Department of Oral Facial Development Indiana University School of Dentistry

Abstract

Objective—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 (CP). Animal models are particularly useful tools for dissecting underlying genetic components of CP. We describe a new CP model resulting from a transgene insertion mutation.

Results—Transgene insertion mutagenesis disrupts the genomic organization and expression of the *Ap2β1* gene located on chromosome 11. This gene encodes the β2-adaptin subunit of the heterotetrameric adaptor protein 2 (AP-2) complex involved in clathrin-dependent endocytosis. Homozygous CP mutant mice express no *Ap2β1* mRNA or β2-adaptin protein, and die during the perinatal period. Heterozygous mice are phenotypically normal despite expressing diminished β2-adaptin mRNA and protein compared to wildtype. Remarkably, the paralogous β1-adaptin subunit of the AP-1 complex partially substitutes for the missing β2-adaptin in embryonic fibroblasts from homozygous mutant mice, resulting in assembly of reduced levels of an AP-2 complex bearing β1-adaptin. This variant AP-2 complex is therefore apparently capable of maintaining viability of the homozygous mutant embryos until birth, but insufficient to support palatogenesis.

Address correspondence to: Eric T. Everett, Ph.D., Department of Pediatric Dentistry, 228 Brauer Hall, CB# 7450, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, U.S.A., Phone 919-425-3565, FAX 919-425-3530, eric_everett@dentistry.unc.edu.
[#]contributed equally to this study
[*]Present Addresses: Wei Li, Ph.D., Research Faculty, Department of Pathology and Laboratory Medicine, Indiana University School of Medicine
Rosa Puertollano, Ph.D., Principal Investigator, Laboratory of Cell Biology, National Heart, Lung, and Blood Institute (NHLBI), National Institutes of Health (NIH)
Eric T. Everett, Ph.D.*, Associate Professor, Department of Pediatric Dentistry, University of North Carolina at Chapel Hill

The authors state that they have no conflicts of interest.

Conclusion—Non-syndromic CP in an animal model is associated with disruption of the *Ap2β1* gene.

Keywords

cleft palate; mouse; transgene insertion mutagenesis; β2-adaptin; clathrin coated pits

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 occurs when there is transformation of the medial edge epithelia (MEE) along with remodeling of the extracellular matrix (ECM). Perturbation of this complex cascade of events can lead to cleft palate (CP)(Ferguson, 1988). Non-syndromic CP (i.e. no other recognizable defects or disabilities) represents a common clinical outcome of genetically heterogeneous etiologies. Growing number of genes encoding transcription factors, signaling molecules, growth factors, growth factor receptors, and extracellular matrix proteins have been implicated in the pathogenesis of syndromic and non-syndromic orofacial clefts (Schutte and Murray, 1999; Chong, et al., 2002; Lidral, et al., 2008; Marazita, et al., 2009; Shi, et al., 2009). Transgene insertion mutagenesis is a common occurrence during the generation of transgenic mice (Meisler, 1992) and has led to the identification 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 ($α$, $γ$, $δ$, or $ε$ and $β1$, $β2$, or $β4$, respectively) that are linked to a medium chain ($μ1$, $μ2$, $μ3$, or $μ4$) and one small chain ($σ1$, $σ2$, $σ3$, or $σ4$). The AP-2 adaptor complex ($α$, $β2$, $μ2$, and $σ2$) is key to successful clathrin dependent endocytosis from the plasma membrane whereas the AP-1 adaptor complex ($γ$, $β1$, $μ1$, and $σ1$) is associated with the trans-Golgi network and endosomes (Traub, 1997; Traub, 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; β2-adaptin; β-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; β-prime adaptin 1; β1-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 β2-adaptin and β1-adaptin, respectively are used through this present study.

Herein we report the use of transgene insertion mutagenesis to show that disruption of the *Ap2β1* gene encoding the β2-adaptin subunit of the AP-2 complex causes perinatal mortality and non-syndromic CP.

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. ICR females bred to vasectomized BDF1 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 initially made by the presence of coat color and pigmented eyes. The BALB/c promoter driving the tyrosinase 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 was also confirmed by standard PCR using GeneAmp® PCR reagents (Applied Biosystems, Foster City, CA)and TYBS specific primers (TYBS 636F ctgaaatatggagggacattgatt and TYBS1034R tcaaactcagacaaaattccacatt) to amplify a 400-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® Gene Expression Assays. 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® Gene Expression Assays using TaqMan® Universal PCR Master mix (Applied Biosystems), Ap2b1 (Mm00551136_m1), and Pgk (Mm00435617_m1) as an endogenous control.

Genomic library construction and screening

Genomic DNA was prepared from a pool of 3 homozygous OVE427 CP embryos using the Pure™ Tissue DNA Isolation Systems (DNA Technologies, Inc., Gaithersburg, MD). Following partial digestion with *Sau*3AI the DNA was size fractionated using sucrose gradients into two size ranges (9.0–13.0 kb and 13.0–23.0 kb) and then ligated to *Bam*HI 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 8×10^6 clones and unamplified titers of ~1×10^{10} pfu/ml. Aliquots of the unamplified 9.0–13.0 kb and 13.0–23.0 kb libraries were screened using PCR (primers described above). The PCR positive OVE427 library containing 13.0 to 23.0 kb, 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 non-isotopic DNA-DNA hybridization with a 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 18d.p.c. embryos or adult OVE427 +/+ tissues using Tri-Reagent (Molecular Research Center, Cincinnati, OH). Twenty to 35 micrograms 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 pre-hybridized/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 (0.5X SSC / 0.1% w/v 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 (MEFs)

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 4.5g/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% CO2 and passaged by lifting with 0.5g/L trypsin / 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 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 were 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, v/v) for 10 min at −20 C. Incubation with primary antibodies diluted in PBS, 0.1% w/v saponin and 0.1% w/v BSA, was carried out for 1h at room temperature. Unbound antibodies were removed by rinsing with PBS for 5 min, and cells were subsequently incubated with secondary antibody (Alexa555 or Alexa488-conjugated goat anti-rabbit or anti-mouse Ig) diluted in PBS, 0.1% w/v saponin and 0.1% BSA, for 30–60 min 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% v/v Triton X-100) supplemented with a protease inhibitor cocktail, Sigma P-8340 (Sigma-Aldrich Co., St. Louis, MO), centrifuged at 16,000xg for 10 min, and supernatants were collected. For immunoprecipitation, lysates were incubated with 5μl of anti-α (Affinity BioReagents) and protein G-Sepharose (Amersham Bioscience, Piscataway, NJ) at 4°C overnight. Immunoprecipitates were then collected, washed 4 times with PBS, and eluted by incubation with Laemmli sample buffer (2% w/v SDS, 10% v/v glycerol, 5% v/v 2-mercaptoethanol, 0.002% v/v bromophenol blue and 0.0625M Tris HCl, pH 6.8) for 10 min at room temperature. Samples were analyzed by SDS–PAGE (4–20% gradient gels) under reducing conditions and transferred onto nitrocellulose. The membranes were then blocked with 1X PBS, 0.05% v/v Tween-20, 10% w/v non-fat 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 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).

Identification of the Ap2β1 gene as the CP locus

Following *SalI* digestion of a single OVE427 lambda clone, four DNA fragments of approximately 20kb, 9kb, 8kb and 5kb were identified. Southern transfer and hybridization using the TYBS minigene fragment revealed hybridization signals over the 5kb and 8kb bands and not the 9kb and 20kb bands (data not shown). The 9kb and 20kb 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 5kb and 8kb *SalI* bands subcloned into pBluescript was performed. The *SalI* fragments were subjected to secondary digestion using *Xba*I 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 2.3kb 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 β1 subunit) gene (Fig. 1B). From the right arm a 4.2kb 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 ~50kb apart in the *Ap2β1* gene. Sequencing within and between the TYBS transgene in this clone confirmed its presence and indicated that 2 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 ~5.8kb *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 the heterozygote when compared to 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 β2-adaptin protein in cells and tissues from homozygous mutant mice

The *Ap2β1* gene encodes a protein named β2-adaptin that, together with α-adaptin, μ2 and σ2, 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 β2-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 β2-adaptin relative to those from wildtype mice. Immunoblot analysis of wildtype MEFs with two different antibodies that recognize both β2-adaptin and the homologous β1-adaptin subunit of the AP-1 complex showed a doublet corresponding to the faster migrating β2 and the more slowly migrating β1 (Fig. 5A). MEFs from heterozygous mice exhibited reduced levels of β2 and unchanged levels of β1, whereas those from homozygous mutant mice showed only β1 expression (Fig. 5A). Levels of α-adaptin were reduced in homozygous mutant MEFs (Fig. 5A) and tissues (Fig. 5B), suggesting that the absence of β2-adaptin partially destabilizes α-adaptin. In contrast, the levels of the γ-adaptin subunit of AP-1 were unchanged (Fig. 5A). These analyses thus showed that homozygous mutant mice did not synthesize any β2-adaptin and that this deficiency coincided with reduced the levels of α-adaptin.

Partial substitution of β1-adaptin for β2-adaptin in homozygous mutant mice

To examine the assembly status of α-adaptin in MEFs from wildtype, heterozygous and homozygous mutant mice, we performed immunoprecipitation of α-adaptin from cell lysates followed by immunoblotting with an antibody that recognizes both β1 and β2 (Fig. 5C). We found that in wildtype MEFs α co-precipitated almost exclusively with β2, as expected for two subunits of the AP-2 complex (Fig. 5C). Strikingly, α co-precipitated with equal amounts of β2 and β1 in heterozygous cells, and only with β1 in homozygous mutant cells (Fig. 5C). This indicates that β1 can substitute for β2 in the AP-2 complex. The amount of β1 co-precipitated with α in homozygous mutant cells was higher than that in wildtype cells (in which there is β2) (Fig. 5C). This means that under normal conditions α prefers to assemble with β2 over β1, but accepts β1 when there is no β2.

Normal distribution of the variant AP-2 complex containing β1-adaptin

We next examined by immunofluorescence microscopy the distribution of the variant AP-2 complex containing β1-adaptin in wildtype (+/+) and homozygous mutant (Tg/Tg) MEFs. We observed that α-adaptin localized to punctate foci at the plasma membrane corresponding to clathrin-coated pits in both wildtype and homozygous mutant MEFs (Fig. 6, A and B). Interestingly, whereas in wildtype cells β1-adaptin localizes to the *trans*-Golgi network (TGN) (Robinson, 2004 and data not shown), in homozygous mutant MEFs β1-adaptin was found on both the TGN and plasma membrane puncta (Fig. 6, D and G). These puncta, but not the TGN structure, contained another plasma membrane clathrin-associated adaptor, epsin 1 (Fig. 6, C and F), identifying them as clathrin-coated pits and vesicles. From these experiments we concluded that incorporation into the AP-2 complex draws β1 to plasma membrane clathrin-coated pits and that the substitution of β1 for β2 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 non-syndromic cleft palate. 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 β2-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 β2-adaptin function leads to CP is not entirely clear. β2-adaptin is a subunit of the heterotetrameric AP-2 complex involved in clathrin-dependent endocytosis of receptors from the plasma membrane (Kirchhausen, 2002; Robinson, 2004).

It is thus likely that CP in β2-adaptin-mutant mice results from defective endocytosis of one or more receptors or other cell surface proteins involved in palatogenesis. TGF-β 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 medial edge epithelial cells (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-β 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-β receptors depends on the interaction of their cytosolic tails with the β2-adaptin subunit of AP-2 (Yao, et al., 2002). This interaction is in contrast to that of other endocytic receptors, which bind to the μ2 subunit (Ohno, et al., 1995; Boll, et al., 1996; Ohno, et al., 1996) or a combination of the α and σ2 subunits of AP-2 (Chaudhuri, et al., 2007; Doray, et al., 2007; Mitchell, et al., 2008). It is likely that the absence of β2-adaptin impairs internalization of TGF-β 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 β2-adaptin-deficient mice might result in part from perturbation of TGF-β signaling.

Perturbation of GABAergic signaling also leads to cleft palate (Homanics, et al., 1997; Hagiwara, et al., 2003). Interestingly, GABA(A) receptors cycle between the synaptic membrane and intracellular sites, and 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, β1-adaptin-containing AP-2 complex could also contribute to the cleft palate phenotype of the β2-adaptin-deficient mice.

In addition to the substitution of β1-adaptin for β2-adaptin, the lower levels of the variant AP-2 complex (~50%, as inferred from the levels of α-adaptin) in the β2-deficient mice could also be a contributing factor leading to cleft palate. Such substitution of β1-adaptin for β2-adaptin has been observed (Keyel, et al., 2008). Interestingly, heterozygous μ2-deficient mice also have reduced levels of the β2-adaptin AP-2 complex (~70% of α-adaptin levels seen in wildtype) but display no phenotypic abnormalities (Mitsunari, et al., 2005).

Homozygous β2-adaptin-deficient embryos survive development and die perinatally due to CP. This phenotype contrasts sharply with that of homozygous μ2-deficient embryos, which die before day 3.5 p.c. (Mitsunari, et al., 2005). As shown here the reason for the different outcomes likely lies in the unique ability of β1-adaptin to substitute for β2-adaptin in the AP-2 complex. Indeed, mouse β1- and β2-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 β1- and β2-adaptins promiscuously interact with other subunits from AP-1 and AP-2 in co-precipitation and yeast two-hybrid analyses (Page and Robinson, 1995). An extreme case is *D. melanogaster*, in which a single β1/2-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 29–45% amino acid sequence identity and are not interchangeable (Page and Robinson, 1995). Thus, the inability of μ1 to be

incorporated into the AP-2 complex likely accounts for the early embryonic lethality of μ2-deficient (Mitsunari, et al., 2005). The converse is also the case, as μ2 does not assemble into the AP-1 complex in μ1A-deficient mice, resulting in mid-gestation embryonic lethality (Meyer, et al., 2000). Thus, the survival of β2-adaptin-deficient embryos until birth likely results from the rescue of most AP-2 functions by substitution with β1-adaptin.

Acknowledgments

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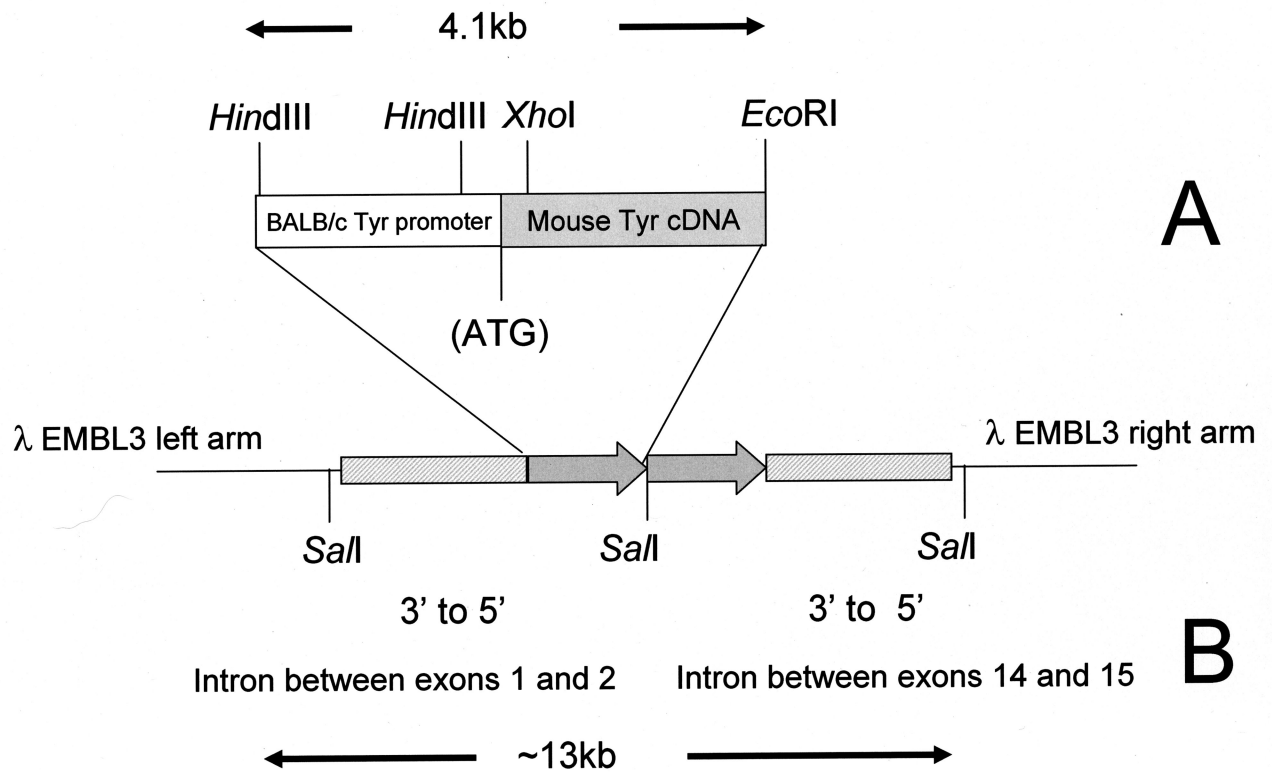
Fig. 1.
Schematic representation of the TYBS transgene and genomic clone. The 4.1 kb TYBS minigene (Panel A) is present as two copies (shaded arrows) in the ~13 kb λ EMBL3 genomic clone (Panel B). Genomic DNA flanking the transgene complex corresponds to intronic sequences between exons 14 and 15 and exons 1 and 2 of the *Ap2β1* gene.
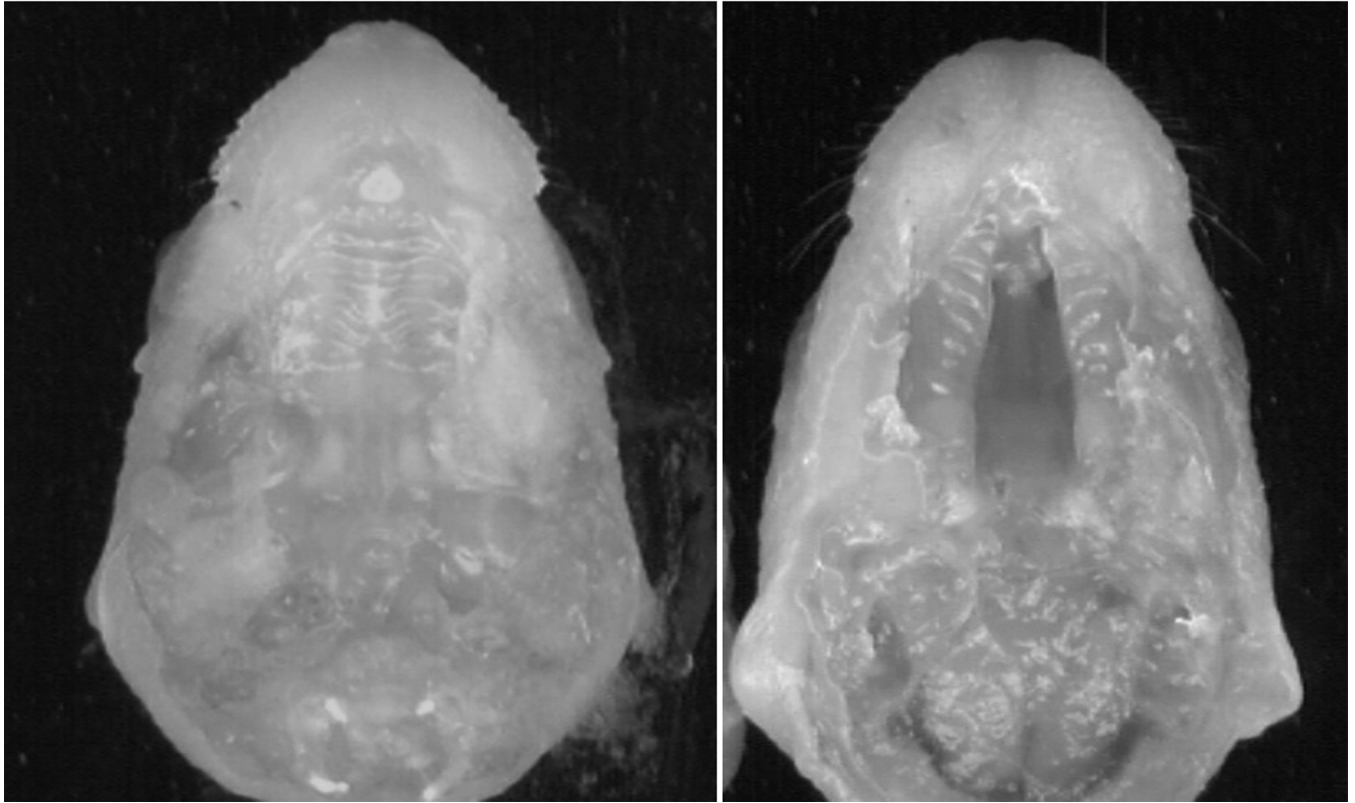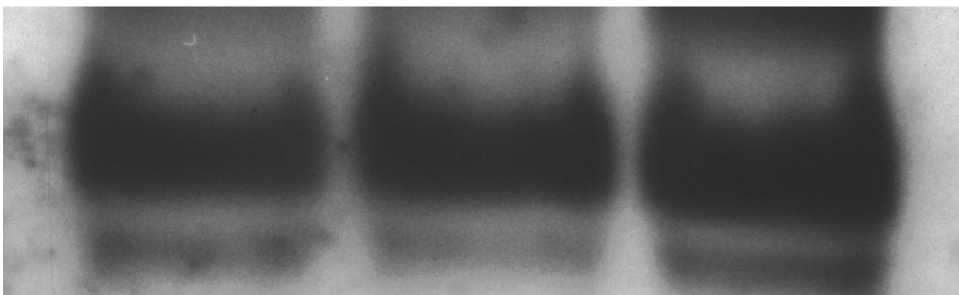
+/+ Tg/Tg

Fig. 2.
OVE427 wildtype and homozygous embryos. OVE427 Tg/+ male and female mice were
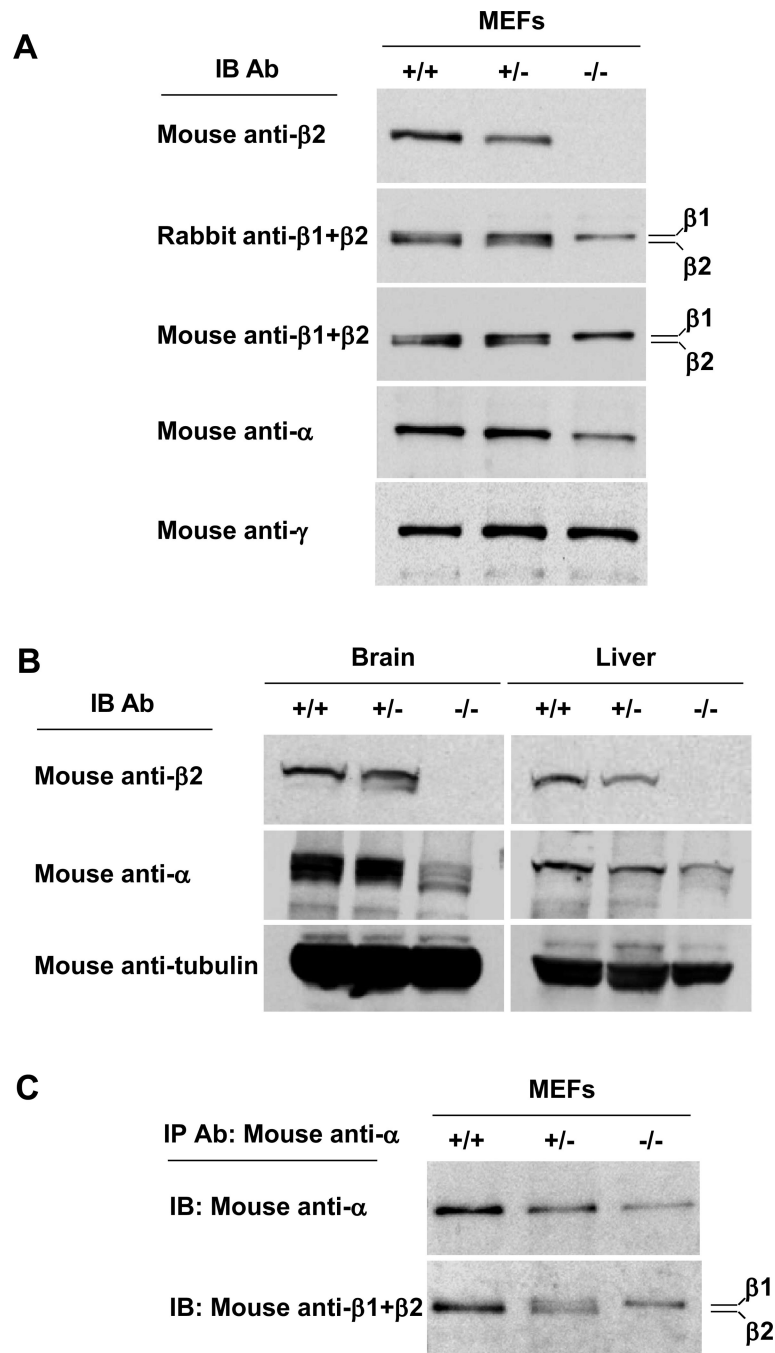mated and 18 d.p.c. embryos collected following cesarean section. CP is present in the
OVE427 Tg/Tg embryo.
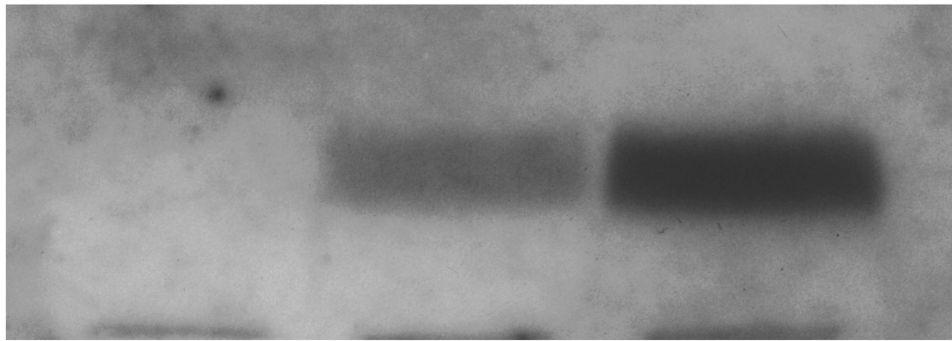
Fig. 3.
Northern blot analysis of mouse *Ap2βb1* and *β-actin* transcripts in total RNA from OVE427
Tg/Tg, Tg/+, and +/+ embryos. Twenty five micrograms of total RNA was blotted in each
lane. The 5.8kb *Ap2β1* transcript is present in heterozygous (Tg/+) and wildtype (+/+) lanes.
After hybridization with a cocktail of *Ap2β1* C-terminal and N-terminal probes the filter
was stripped and hybridized with a murine β-actin probe.
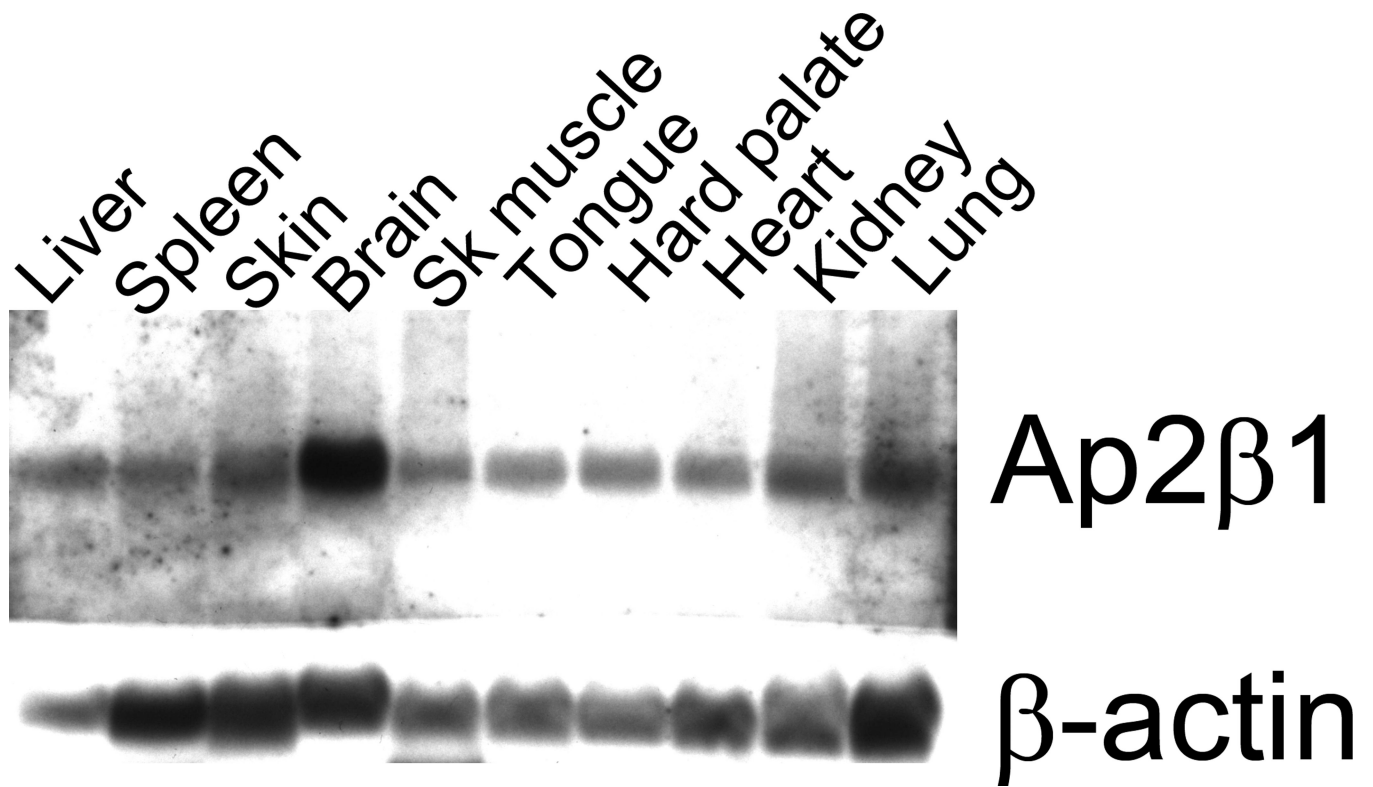
Fig. 4.
Multiple tissue Northern blot analysis of mouse *Ap2β1* and *β-actin* transcripts in total RNA. Thirty-five micrograms of total RNA was blotted in each lane. The blot was hybridized with a cocktail of *Ap2β1* C-terminal and N-terminal probes, stripped, and re-hybridized with a 600-bp murine β-actin probe. Tissues surveyed were the liver, spleen, skin, brain, skeletal muscle, tongue, hard palate, heart, kidney and lung.

A

MEFs

IB Ab

| | +/+ | +/- | -/- |

Mouse anti-β2

Rabbit anti-β1+β2 → β1 / β2

Mouse anti-β1+β2 → β1 / β2

Mouse anti-α

Mouse anti-γ

B

	Brain			Liver		
IB Ab	+/+	+/-	-/-	+/+	+/-	-/-

Mouse anti-β2

Mouse anti-α

Mouse anti-tubulin

C

MEFs

IP Ab: Mouse anti-α

| | +/+ | +/- | -/- |

IB: Mouse anti-α

IB: Mouse anti-β1+β2 → β1 / β2

Fig. 5.
Characterization of the AP-2 complex in Ap2β1 mutant mice. Embryonic fibroblasts (A) or brain and liver tissues (B) 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-α as described in Materials and Methods. Immunoprecipitates were resolved by SDS-PAGE and immunoblotting with antibodies against α (upper panel) or β1+β2 (lower panel).
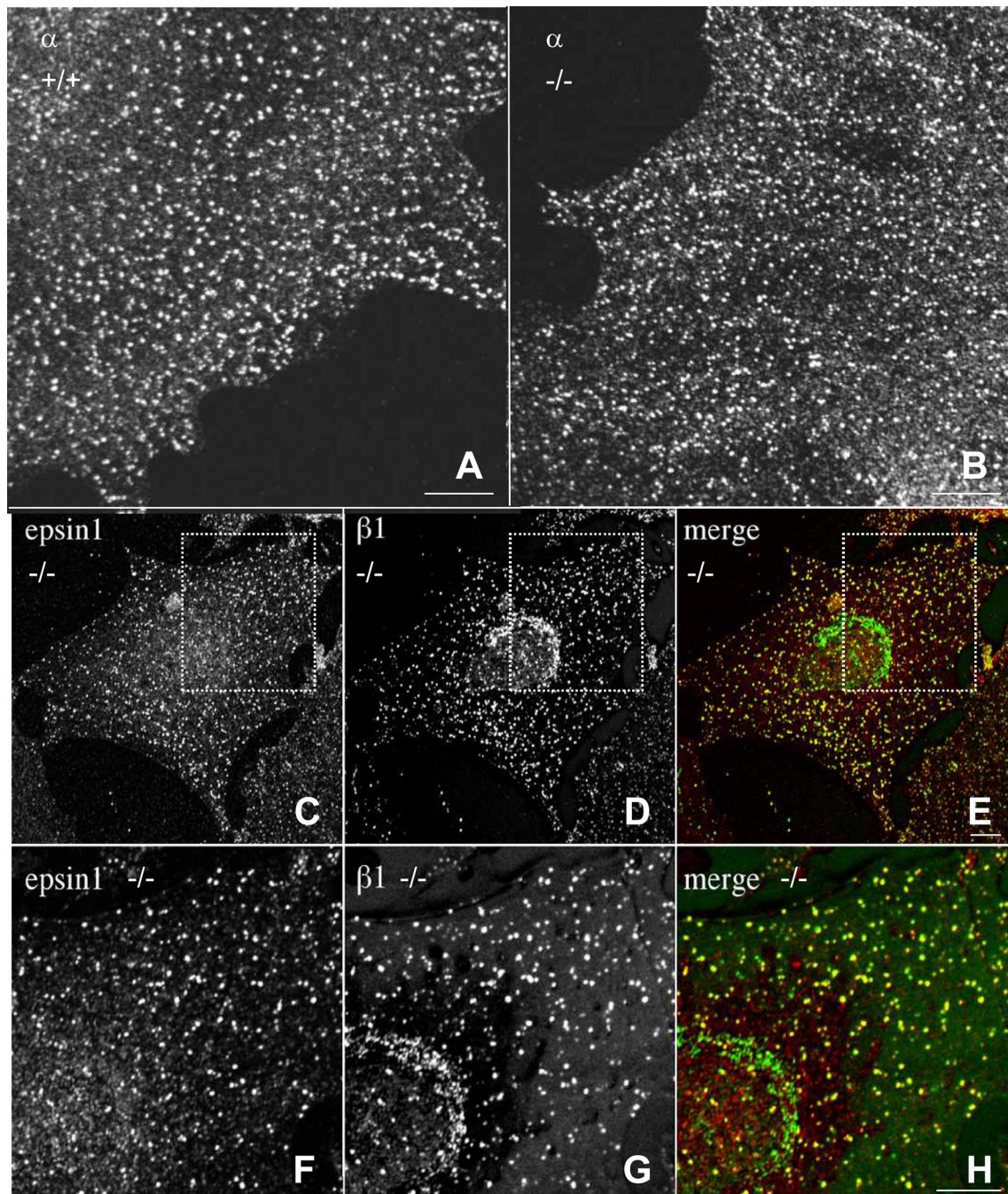
Fig. 6.
β1 subunit is partially incorporated into AP-2 complexes in Ap2β1 mutant mice. (A,B)
Wild-type (+/+) and homozygous (Tg/Tg) mouse embryonic fibroblasts were grown on
coverslips, fixed in methanol:acetone (1:1), and stained with mouse monoclonal antibodies
against α followed by Alexa488-conjugated goat anti mouse IgG. Cells were examined by
confocal fluorescence microscopy. Scale bar, 10 μm. (C-E) (Tg/Tg) fibroblasts were fixed
and double-stained with antibodies against epsin (red) (C) and β1 (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. Panels F, G, and H are two-fold magnification of the regions shown in panels C, D, and E. Scale bar = 10 μm.