The gene defective in fidget mice encodes fidgetin, a member of the AAA family of ATPases. Using a yeast two-hybrid screen, we identified cAMP-dependent protein kinase A anchoring protein 95 kDa (AKAP95) as a potential fidgetin-binding protein. Epitope-tagged fidgetin colocalized with endogenous AKAP95 in the nuclear matrix and the physical interaction between fidgetin and AKAP95 was further confirmed by reciprocal immunoprecipitation. To evaluate the biological significance of the fidgetin-AKAP95 binding, we created AKAP95 mutant mice through a gene-trap strategy. Akap95 mutant mice are surprisingly viable with no overt phenotype. However, a significant number of mice carrying both Akap95 and fidget mutations die soon after birth due to cleft palate, consistent with the overlapping expression of AKAP95 and fidgetin in the branchial arches during mouse embryogenesis. These results expand the spectrum of the pleiotropic phenotypes of fidget mouse and provide new leads on the in vivo function of AKAP95.

The fidget mutation is an autosomal recessive mutation (1) that causes multiple developmental defects, including reduced or absent semicircular canals (2), insufficient growth of the retinal neural epithelium (3) and abnormal bone development (2). A viral retrotransposon insertion in the second intron of the fidgetin gene (Fign) was identified as the molecular lesion, interfering with the normal splicing of the Fign transcript and hence leading to significantly reduced expression of this gene (4).

Fidgetin is the first AAA protein whose mutation leads to mammalian developmental abnormalities. Robust expression of fidgetin was observed in the sites of defects seen in the affected mice (otocyst, optic cup and pelvic anlage) from mid- to late gestation, indicating a putative role in embryogenesis (4). Mouse fidgetin contains a D>S substitution within the highly conserved AAA domain, suggesting that it is not an ATPase based on computational modeling (4,5). Recently, a C. elegans fidgetin ortholog, F32D1.1, was shown to possess ATPase activity with cysteine residue 368 being critical for ATP hydrolysis (8). However, F32D1.1 does not possess the D>S substitution at the correlating position, therefore whether fidgetin is an ATPase remains to be further investigated. Recent results indicated that fidgetin is a nuclear protein with the potential to form homo-oligomers (9). Despite these efforts, the cellular function of fidgetin and hence the biochemical disease mechanism of this mutation still remain unknown.

Since it has been proposed that the specific function of a given AAA protein is primarily dependent upon the identity of its interacting partner(s) (10), we sought to identify proteins that
bind to fidgetin through a yeast two hybrid screen. We describe here that AKAP95 specifically interacts with fidgetin in yeast and mammalian cells. AKAP95 (mouse gene symbol Akap8, A kinase anchor protein 8) is a nuclear protein (11) that colocalizes with fidgetin in the nuclear matrix. Furthermore, AKAP95 shows a broad expression pattern overlapping with that of fidgetin during mouse embryogenesis. Finally, whereas the Akap95 mutation does not lead to obvious abnormality, deficiency in fidgetin and AKAP95 predisposes mouse embryos to cleft palate, thus establishing one aspect of their functional interaction in vivo.

Experimental Procedures

Yeast Two-hybrid Screening- A region in fidgetin (residue 204~372) was fused in-frame with LexA DNA binding domain as a bait in the plasmid pEG202-NLS (OriGene, Rockville, MD). A mouse embryonic day 11.5 cDNA library was cloned into pYESTrp2 vector (Invitrogen, Carlsbad, CA). Approximately 8 million transformants were screened on a nutritionally selective medium deficient in Ura, His, Leu and Trp. Auto-activation tests and specificity tests were performed according to manufacturer’s recommendation (OriGene).

Plasmid Constructions- The expression vectors pCMV-HA, pCMV-EGFP and pCMV-FLAG were used to drive the expression of all fusion proteins (9). For the plasmid encoding AKAP95-FLAG, the Akap95 cDNA was first generated through PCR. Then both the PCR product and the vector were digested with ClaI and XhoI and subsequently ligated so that the Akap95 open reading frame was inserted in-frame 5’ to the FLAG tag. Other plasmids were constructed analogously and sequenced to be correct.

Cell culture, Transfection and Isolation of Nuclear and Cytoplasmic Proteins- NIH/3T3 cells (ATCC, Manassas, VA) were seeded in 6-well clusters (Corning, Corning, NY) at 1.5\(\times\)10⁵/ml 18 hours prior to transfection. 2 ug DNA (1ug of each construct) was co-transfected using Lipofectamine Plus (Invitrogen). Cells were collected for immunoprecipitation or immunostaining 24 hours afterwards. Differential isolation of nuclear and cytoplasmic proteins was achieved through the NE-PER extraction kit (Pierce, Rockford, IL).

Immunofluorescence Microscopy- Cells were fixed with 2% paraformaldehyde for 15’ on ice and permeabilized for 15’ with ice-cold 4% Triton X-100 in PBS. Fixed cells were incubated with anti-AKAP95 rabbit polyclonal antibody for 1h at room temperature (Upstate, Lake Placid, NY, 1:333), followed by incubation at room temperature for 1h with Alexa Fluor 546 conjugated goat anti-rabbit antibody (Invitrogen 1:500). After several washes, coverslips were directly mounted on the glass slides with PBS containing 90% glycerol, 4% n-propyl gallate, and 1.5 ug/ml DAPI. Slides were examined under a Nikon fluorescence microscope (Eclipse E6000) using a 20X objective.

Immunoprecipitation- Approximately equal numbers of transfected cells were lysed in lysis buffer (50mM Tris pH 7.5, 1% NP40, 150mM NaCl, 1mM DTT, 4mM MgCl₂, 1mM ATP and 5% glycerol) with 1X complete protease inhibitor cocktail (Roche, Indianapolis, IN) and subsequently centrifuged at 13,000 rpm for 15 minutes at 4 °C. The lysates were pre-cleared by protein A/G plus agarose beads (1:25, Santa Cruz Biotechnology, Santa Cruz, CA) for 30’ at 4 °C. Then immunoprecipitation was carried out with fresh protein A/G plus agarose beads (1:25) and anti-HA (1:500, BAbCO, Berkeley, CA) or anti-FLAG M2 (1:500, SIGMA, St. Louis, MO) antibodies, respectively for 3h at 4 °C. The beads were washed three times in cold lysis buffer without the protease inhibitors and washed once with cold 1X PBS. The proteins were released from the agarose beads by boiling in protein gel loading buffer (62.5mM Tris-HCl, pH 6.8, 2% SDS, 25% glycerol, 0.25% (w/v) bromophenol blue and 5% (v/v) -Mercaptoethanol) and then loaded on SDS-PAGE gels.

Western Blot- Protein extracts were resolved by SDS/10% PAGE Ready gel (Bio-Rad, Hercules, CA), transferred to nitrocellulose membrane (Schleicher & Schuell, Keene, NH), probed with the primary antibody and a secondary peroxidase-conjugated antibody and visualized with the ECL plus kit (Amersham, UK).
prestained protein ladder from Invitrogen was used as a reference for molecular mass estimation. Where indicated, the nitrocellulose membrane was incubated with Restore Western blot stripping buffer (Pierce) at 37 °C for 30’ to remove all the antibodies. The membrane was washed and subsequently reprobed with a different set of antibodies. Cell extracts were made from mouse E11.5 whole embryos. Embryos were homogenized in lysis buffer (50mM Tris pH 8.0, 1% NP40, 150mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS) with 1X complete protease inhibitor cocktail (Roche) and subsequently centrifuged at 7,000 rpm for 5 minutes at 4 °C. The supernatant was collected and stored at −80 °C until use. The primary antibodies and the dilutions were: anti-β-galactosidase polyclonal antibody (1:80, Clontech, Mountain View, CA), anti-HA monoclonal antibody (1:1,000, BABCO), anti-FLAG M2 monoclonal antibody and anti-α-tubulin monoclonal antibody (both 1:1,000, SIGMA), anti-AKAP95 antibody (1:666, Upstate). The secondary antibodies and the dilutions were: HRP-conjugated anti-mouse antibody (1:12,000, Invitrogen) and HRP-conjugated anti-rabbit antibody (1:2,000, Amersham).

ES Cells and Mice- A feeder-independent ES cell line (XG068) derived from the 129/Ola mice was obtained from BayGenomic (UC-Davis). Microinjection of the ES cells into blastocysts was carried out by The Jackson Laboratory Microinjection Services. Chimeras were initially crossed to C57BL/6J mice to test germ-line transmission based on coat color. F1 progeny carrying the gene-trap allele were backcrossed to 129S1/SvImJ for 3 generations before analysis. All animal procedures followed AAALAC guidelines and were approved by institutional ACUC.

Genotyping- The genotypes of all mice were confirmed by PCR. Mouse tail DNA was amplified 35 cycles (15 sec, 95 °C; 30 sec, 57 °C; 90 sec, 72 °C). The reaction condition for the fidget allele has been described (4). In order to follow the Akap95 gene-trap allele, three primers were used in the genotyping PCR reaction. 95U1 (part of the eighth exon of Akap95) CAGCCTGAGTGCAAGGCCTTAG, 95D2 (part of the eighth intron of Akap95, 3’ to the gene-trap insertion site) AAATGGGAAGAGACCGACTGGTC and TRAPD (about 650nt 3’ to the En2 splice acceptor site in the gene-trap construct) GTTATCGATCTGCAGTACTGCG. 95U1 and 95D2 generated a 607 nt product from wild-type allele whereas 95U1 and TRAPD amplified an approximately 1.1kb fragment from the Akap95 mutation due to the gene-trap insertion.

β-galactosidase Histochemistry- Dissected embryos from timed matings were fixed in 4% paraformaldehyde for 1 h at room temperature, washed three times in wash buffer (0.1 M phosphate buffer (pH 7.3), 2 mM MgCl2, 0.01% Na-deoxycholate, 0.02% NP-40) and incubated at 37 °C overnight in a stain solution containing Bluo-Gal (1 mg/ml; Invitrogen), K-ferricyanide (5 mM) and K-ferrocyanide (5 mM) in wash buffer. A blue precipitate was seen in embryos carrying the gene-trapped allele, whereas no staining was seen in non-carriers. Embryos were transversely embedded in paraffin, serially sectioned (6 µm), and counterstained with neutral fast red. Sections were imaged on a Nikon eclipse microscope using a Spot RT camera.

Real-time PCR- RNA was prepared from E11.5 embryos with Trizol (Invitrogen). Following DNase I digestion, 2µg RNA was reverse transcribed with AMV reverse transcriptase. The cDNA was diluted five fold and 1 ul was added to Sybr Green PCR mix (Eurogentec, San Diego, CA) with following primers: actinF, CATTGCTGACAGGATGCAGAA; actinR, GCCACCGATCCACACAGAGT; HA95F1, TGATGGAGGACGTCCAAGAA; HA95R1, GGATTCTCGCCCCCTACAGATA; and HA95F2, CATGAAGCGAGATGAAGGGGT; HA95R2, TAGCTTTGCTGTCTGCTCA. The PCR amplifications were monitored by an ABI Prism 7000 sequence detector (Applied Biosystems, Foster City, CA). The PCR reactions were run in triplicate with two separate runs. The cDNA was made from three mutants and three sex-matched wildtype littermates. The correct PCR amplification was confirmed by the dissociation curve function of the ABI machine and by agarose gel electrophoresis.
Skeletal Analysis - Dissected upper jaws of newborn mice were fixed in ethanol for 3 days. The samples were then placed in acetone for 1 day. Skeletons were stained by alcian blue/alizarin red for 3 days (12). The samples were cleared by 1% KOH and a glycerol gradient.

RESULTS

Fidgetin interacts with AKAP95 in yeast. A yeast two-hybrid screen was performed to identify fidgetin-interacting proteins. One particular N-terminal region which did not show significant auto-activation (residues 204~372) was used as a bait against a library of mouse E11.5 cDNAs because of fidgetin’s strong expression at this stage of embryogenesis (4). One of the cDNA clones recovered from the screen contained the majority of the Akap95 open reading frame. AKAP95 did not interact with several control baits (pEG202-Max, pBait, pEG202-NLS and pRHFM1), indicating the specificity of the fidgetin-AKAP95 binding in yeast.

Fidgetin interacts with AKAP95 in mammalian cells. AKAP95 was originally cloned as a nuclear anchoring protein for the regulatory subunit of type II cAMP-dependent protein kinase (RII/PKAII) (13). AKAP95 has also been shown to be involved in mitotic chromosome condensation (14,15). AKAP95 was localized to the nucleus in interphase and was excluded from nucleoli indicating a nuclear matrix distribution (11,13). Recently fidgetin was shown to have a similar intracellular localization (9), prompting us to examine the potential overlapping nuclear distribution between fidgetin and AKAP95. Detection of the endogenous fidgetin was not possible due to the inability to develop a specific antibody despite eight attempts. We therefore expressed epitope-tagged fidgetin in cell lines through transfection. GFP-tagged fidgetin colocalized with endogenous AKAP95 in the nuclear matrix of NIH-3T3 cells (Fig. 1).

To further evaluate the fidgetin-AKAP95 interaction in mammalian cells, we carried out reciprocal immunoprecipitation using cell extracts containing epitope-tagged fidgetin and AKAP95. FLAG-tagged AKAP95 was co-immunoprecipitated with HA-tagged fidgetin by anti-HA antibodies, indicating the formation of fidgetin-AKAP95 complex (Fig. 2). Conversely, HA-tagged fidgetin was precipitated by anti-FLAG antibodies only when FLAG-tagged AKAP95 was present (Fig. 2).

We used deletion constructs to determine the fidgetin region responsible for AKAP95 binding. Full-length fidgetin was split into two parts (at residues 372-373) with approximately equal number of amino acids. Surprisingly, both deletion constructs retained the ability to interact with AKAP95, suggesting the presence of multiple potential AKAP95 binding domains in fidgetin (data not shown). We used the same strategy to map the fidgetin interaction domain in AKAP95 (split at residues 376-377). Only removing the C-terminal half of AKAP95 abolished the fidgetin-AKAP95 interaction (Fig. 3). Together these data suggest that AKAP95 interacts with fidgetin through its C-terminus in mammalian cells.

Disruption of AKAP95 expression by a gene trap in mice. To evaluate the fidgetin-AKAP95 interaction in vivo, we sought to analyze the phenotype of mice deficient in both AKAP95 and fidgetin. We first created AKAP95 mutant mice through an ES cell line where a gene-trap construct inserted into the Akap95 gene (XG068, Baygenomics). The sequence tag associated with the XG068 ES cells matched exons 5 through 8 of Akap95, suggesting that the insertion was in intron 8 (Fig. 4a). Indeed, an intron 8 primer and a pGT1Lxf vector primer amplified an approximately 1.1kb band from the ES cell DNA, confirming the presence of the gene-trap vector in intron 8 (Fig. 4b). Hereafter, we use Akap95GT to denote this mutation where β-galactosidase-neomycin fusion replaces the C-terminal 334 amino acids of AKAP95. The truncation generated from the Akap95GT allele would remove the functional domains responsible for PKA regulatory subunit binding (13) and DNA condensation and chromosome binding (14). Importantly, the AKAP95 truncation would also delete the domain responsible for fidgetin binding (Fig. 3).

Akap95GT homozygotes are viable and show no overt phenotype on a mixed 129;B6 strain background. To confirm that the expression of AKAP95 protein was disrupted in Akap95GT mice, we probed cell extracts made from E11.5 embryos using an AKAP95 antibody (13). Because the insertion was in the eighth intron, a fusion protein
containing the first 354 amino acids of AKAP95 and β-galactosidase-neomycin fusion would be made from the trapped allele. The polyclonal AKAP95 antibody was generated by a rat AKAP95 fragment corresponding to amino acid residues 377-687 of mouse AKAP95, therefore, the epitopes would be missing in Akap95<sup>GT</sup> homozygotes. As predicted, robust signal was present in both wildtype and heterozygous embryo extracts, but absent in homozygous samples (Fig. 5). We determined that there was approximately 50% reduction in AKAP95 abundance at this embryonic stage in heterozygotes (heterozygote/wildtype=0.51, n=5, S.D.=0.21).

**Examination of AKAP95 and HA95 in gene trap mice** Since AKAP95 has been implicated in many important biological processes, it is surprising that Akap95<sup>GT</sup> homozygotes do not have overt abnormalities. We performed two sets of control experiment to examine the expression of AKAP95 and HA95 (homologous to AKAP95), a molecule with 61% homology to AKAP95.

The gene-trap inserted into the eighth intron, therefore the first 354 amino acids of AKAP95 would still be present in the Akap95<sup>GT</sup> mice. Several reports indicated that, through its N-terminus, AKAP95 interacts with other proteins including minichromosome maintenance 2 protein (MCM2) (16) and p68 RNA helicase (p68) (11). To examine the expression of the AKAP95-β-galactosidase-neomycin fusion protein, we probed the nuclear and cytoplasmic cell extracts from E11.5 Akap95<sup>GT</sup> heterozygous embryos with anti-β-galactosidase antibodies and anti-AKAP95 antibodies. Although AKAP95 is localized to the nuclear fraction, the fusion protein is primarily cytoplasmic (Fig. 6). We note that there are multiple predicted nuclear localization signals (NLS) in AKAP95 and most are still present in the fusion protein. Therefore, it appears that the fusion tag (β-galactosidase-neomycin) with a predicted molecular mass of >120 kDa contributed to the cytoplasmic distribution of the chimeric protein, thus anchoring the N-terminus of AKAP95 away from the nucleus where it is thought to function.

HA95 is a nuclear protein with 61% homology to AKAP95 (17). While HA95 and AKAP95 were reported to have distinct cellular functions (17,18), the gene encoding HA95 is immediately distal to the Akap95 gene on mouse Chr. 17. We examined whether the AKAP95 gene trap insertion interfered with the expression of HA95, but no differences were observed (Table 1).

**Akap95 is expressed widely during mouse midgestation.** We used the β-galactosidase fusion tag embedded in the gene trap construct to characterize gene expression in embryos. Previously, strong fidgetin expression was observed from mid- to late-gestation in key sites consistently affected in fidget mice, namely the otocyst, optic cup and pelvic anlage (4). Akap95 showed a strong expression throughout the entire E11.5 embryos (Fig. 7a). In particular, Akap95 expression in the branchial arches (Fig. 7b), retinal neural epithelium and otocyst (data not shown) overlapped with that of fidgetin.

**Defects in AKAP95 and fidgetin predispose mice to cleft palates.** We backcrossed Akap95<sup>GT</sup> heterozygotes to 129S1/SvImJ congenic fidget mice and then intercrossed the progeny to generate mice homozygous for both Akap95 and fidget mutations. Only three of the 258 mice analyzed at weaning were double homozygotes (32 expected). The surviving double mutants were small with eye defects and circling behavior reminiscent of fidget mice. Further studies revealed that at E11.5 and E18.5, Mendelian ratios of homozygotes were observed (data not shown). Double mutants were indeed born alive but failed to thrive. There appeared to be two waves of loss in double mutants. The first wave was right after birth with nearly half of the mice surviving only a few hours. The remaining were always smaller than their littermates, did not nurse well and succumbed to death gradually before wean. Some newborn double homozygotes were gasping, retaining a blue skin color before death. We analyzed 172 newborn mice from various crosses and found that 50% of double mutants showed a cleft palate phenotype (Table 2), consistent with the overlapping expression of AKAP95 and fidgetin in branchial arches during mouse embryogenesis. It is interesting to note that approximately 20% of the animals with fidget mutation alone on the same mixed 129;B6 background had cleft palate while removing one copy of Akap95 did not seem to change the cleft palate rate. The cleft palate
phenotype has not been observed in fidget mice maintained on B6 background, indicating that the presence of 129 alleles is required. The palate defect in the double mutant was complete cleft of the secondary palate (Fig. 8), the same phenotype observed in fidget animals with cleft palate on the mixed 129;B6 background (data not shown). Taken together, our results suggest that removing the C-terminus of AKAP95, hence abolishing AKAP95-fidgetin interaction, significantly exaggerated the cleft palate phenotype caused by fidgetin deficiency (Table 2, p=0.004, Fisher’s exact test).

DISCUSSION

Fidgetin is a member of subfamily-7 of AAA proteins which does not seem to have a set of unifying functions (7). AAA proteins are ATP-dependent molecular chaperones that function through protein-protein interactions. It has been proposed that the function of a given AAA protein is defined in part by its binding partner and in part by the subcellular localization of the interaction (10). Through a yeast two-hybrid screen, we identified a fidgetin-interacting protein, AKAP95. In vitro studies confirmed that AKAP95 specifically interacted with fidgetin through its carboxyl-half. During mouse embryogenesis, AKAP95 expression spatially and temporally overlapped with that of fidgetin. We observed low-penetrance cleft palate in fidget mice maintained on a mixed 129;B6 background, adding a new facet to the pleiotropic fidget phenotypes. Consistent with the strong expression of both molecules in the maxillary component of the branchial arches, deficiency in AKAP95 and fidgetin significantly increased (more than 2-fold) the incidence of cleft palate in the double mutants, establishing a non-redundant role for the AKAP95-fidgetin interaction in vivo.

AKAP95 binds to many proteins such as the regulatory subunit of type II cAMP-dependent protein kinase (13); Eg7, a chromosome condensing complex component (14); p68 RNA helicase (11); a novel c-Myc-binding protein (19); minichromosome maintenance 2 protein (16); D-type cyclins (20) and phosphodiesterases (21). Thus AKAP95 has been proposed to be involved in a variety of cellular functions including PKA signaling, DNA condensation, transcription and DNA replication (22). Recent reports indicate that AKAP95 is also involved in cAMP-regulated mRNA stability (23) and the nuclear translocation of active caspase 3 (24). Despite numerous attempts, no in vivo role has been established for AKAP95 yet.

We now show that replacing AKAP95’s C-terminal 334 residues with a [-galactosidase-neomycin fusion protein does not cause overt abnormalities in mice. It is important to note that the C-terminus of AKAP95 replaced by the fusion tag from the Akap95GT allele includes functional domains responsible for PKA regulatory subunit binding, DNA condensation, chromosome binding and caspase 3 binding. More importantly for our study, the AKAP95 truncation would prevent itself from binding to fidgetin (Fig. 3). Still, the remaining N-terminus of AKAP95 possesses putative NLS’s and the potential to interact with nuclear proteins such as MCM2 (16) and p68 (11). However, we also show that the fusion protein is predominantly cytoplasmic where the N-terminus of AKAP95 may not carry out its normal nuclear function, indicating that the Akap95 gene trap allele abolishes most if not all proposed functions of AKAP95 in the mouse. We also found no evidence for compensation at the expression level by the related gene HA95, suggesting that AKAP95 is functionally redundant, at least in laboratory mice on a mixed 129; B6 background.

However, when both AKAP95 and fidgetin are deficient, the double mutants show elevated incidence of cleft palate, suggesting that the specific AKAP95-fidgetin binding could not be compensated by other mechanisms. Although the fusion protein between the N-terminal AKAP95 and [-gal-Neo generated by the Akap95GT allele has the potential to cause phenotype(s) through a dominant-negative fashion, multiple lines of evidence suggest that the cleft palate phenotype(s) was not due to the fusion protein. First, mice carrying either one or two copies of Akap95GT did not show overt phenotypes. Second, we did not observe significant difference in cleft palate incidence between fidget mice with one copy of the Akap95GT and fidget mice with wildtype Akap95 (95gt/+, fi/fi, 6/31 and +/+; fi/fi, 7/37). Third, gene trap homozygotes did not show elevated incidence of cleft palate (95gt/95gt, fi/+,
1/50 and 95\textsuperscript{th}/95\textsuperscript{th}, +/+, 0/20). Together these observations point to a critical role for the fidgetin-AKAP95 interaction in mouse palatogenesis.

What is the disease mechanism leading to cleft palate in the double mutants? Most mouse mutations with developmental phenotypes are caused by defects in signaling pathways involved in embryogenesis. As a result, mutations (either spontaneous or targeted) are frequently found in upstream signaling molecules including morphogen and morphogen receptor or in downstream transcription factors. In this regard, both fidgetin and AKAP95 are exceptions, in that they are molecular chaperones/scaffolding proteins. Recently, a new trend is emerging where mutations associated with developmental defects were found in regulatory molecules other than the actual components of the pathway (25,26). For example, mutations in intraflagellar transport proteins can cause defects in Sonic hedgehog (Shh) signaling in mouse embryonic development through cilia formation, intracellular transport or a combination of both mechanisms (26). Based on the cleft palate phenotype, we speculate that the fidgetin-AKAP95 interaction may modulate signaling pathway(s) underlining palatogenesis in mice, with cAMP-dependent protein kinase (PKA) being a likely candidate. To direct and amplify the cAMP signaling, the regulatory subunits of PKA and hence the holoenzyme are targeted and compartmentalized to discrete subcellular locations by AKAPs (27). PKA is a negative regulator of Shh signaling (28-30) and germline removal of GLI2, a Shh target, also resulted in high incidence of cleft palate in mice (31). It is interesting to note that some of the phenotypes in fidget mice such as small eyes and absent semicircular canals are also found in mice deficient in Shh (32) and GLI3, another Shh target (33), suggesting that Shh signaling may be involved in the defects demonstrated by fidget and fidgetin-AKAP95 double mutants.

In humans, cleft lip and/or palate is a common birth defect with both genetic and environmental contributions. Despite intensive efforts, the major genes responsible for cleft lip and/or palate still remain elusive. The best fit genetic model has been predicted to be an oligogenic one where a few major susceptibility genes interact with a small number of modifier genes (34). Nine human chromosome regions have been implicated in cleft lip and/or palate to date (35). In humans, the FIDGETIN gene is on 2q24 and the AKAP95 gene is on 19p13, both chromosomes contain critical regions for cleft lip and/or palate (35). While the FIGN and AKAP95 genes are >30 Mb from the leading candidate genes in the respective critical region, further investigation of the fidgetin-AKAP95 interaction may nevertheless provide a better understanding of human cleft palate pathogenesis.

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FOOTNOTES
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The abbreviations used are: AAA, ATPases associated with diverse cellular activities; AKAP95, cAMP-dependent protein kinase A anchoring protein 95 kDa; B6, C57BL/6J; ES cells, embryonic stem cells; GFP, green fluorescent protein; HA95, homologous to AKAP95; PKA, cAMP-dependent protein kinase; Shh, sonic hedgehog.

FIGURE LEGENDS
Figure 1. Co-localization of Fidgetin-GFP and AKAP95 in transfected cells. NIH-3T3 cells were transiently transfected with a plasmid encoding fidgetin-GFP and immunostained to reveal the intracellular localization of the endogenous AKAP95 protein. Representative Fidgetin-GFP (panel A), AKAP95 (panel B) and DAPI (panel C) images of the same magnification field are shown. Note that both the Fidgetin-GFP fusion protein and AKAP95 seemed to be excluded from the nucleoli.
Figure 2. Co-immunoprecipitation of fidgetin-HA and AKAP95-FLAG. A. Cell extracts containing both fidgetin-HA (Fign-HA) and AKAP95-FLAG (95-FLAG) and those containing AKAP95-FLAG only (lane 2) were precipitated with anti-HA antibodies, separated by SDS-PAGE and subsequently probed with anti-FLAG antibodies. Approximately equal amount of 95-FLAG was present in the starting material as shown in lane 1 and lane 2. The interaction between Fign-HA and 95-FLAG was specific as the anti-HA antibodies did not precipitate 95-FLAG without Fign-HA as shown in lane 4. B. The experiments were carried out in the reverse direction with anti-FLAG antibodies as precipitating antibodies and anti-HA antibodies as detecting antibodies in the western blot. Lane 2 contained Fign-HA only and the anti-FLAG antibodies did not pull down Fign-HA without 95-FLAG in lane 4.

Figure 3. AKAP95 interacts with fidgetin through its C-terminal half. Cell extracts containing fidgetin-HA (Fign-HA) and AKAP95-FLAG deletion constructs together with anti-FLAG immunoprecipitants were probed with anti-HA antibodies. Full-length 95-FLAG interacted with Fign-HA (lanes 1 and 5). Deleting the C-terminal half abolished the binding (lanes 2 and 6) whereas removing the N-terminal half had no effect (lanes 3 and 7).

Figure 4. Gene trap insertion at the Akap95 locus. A. Physical map of the gene trap insertion. The pGT1Lxf vector landed into the eighth intron of Akap95 gene. The usage of the En2 (engrailed 2) splicing acceptor site in the vector would generate a fusion message between Akap95’s first eight exons and β-geo (β-galactosidase-neomycin resistance gene fusion) cDNA, truncating wildtype AKAP95 after amino acid residue 354. Arrows represent PCR primers used for genotyping. pA, polyadenylation signal; U, 95U1; D, 95D2; TD, TRAPD. B. PCR detection of the gene trap mutation. Genotyping PCR reaction using three primers was carried out as described in Experimental Procedures. Note the homozygote (lane 3) only had the 1.1 kb PCR product whereas the wildtype (lane 1) only had the 607 nt product. Both bands were present in the heterozygotes (lanes 2 and 4). Lane 5, X174/Hae III markers. hom., homozygote; het., heterozygote.

Figure 5. Absence of full-length AKAP95 protein in the Akap95GT homozygotes. Mouse E11.5 embryo cell extracts were probed by anti-AKAP95 antibodies that recognized epitopes encoded by cDNA 3’ to the gene trap insertion site. The membrane was stripped and reprobed with anti-β-tubulin antibodies to verify equal loading. Note the reduced expression in the heterozygote. hom., homozygote; het., heterozygote.

Figure 6. The Akap95GT allele encodes a cytoplasmic protein. The nuclear and the cytoplasmic fraction of mouse E11.5 embryo cell extracts were sequentially probed with anti-β-gal and anti-AKAP95 antibodies. Cell extracts were made from two heterozygous mice. Cy, cytoplasmic fraction; Nu, nuclear fraction.

Figure 7. Expression of Akap95 in homozygous Akap95GT mice. A. Left lateral view of an E11.5 embryo showing broad lacZ staining. B. Representative transverse sections of mouse embryos at E11.5, showing the overlapping lacZ staining pattern in the maxillary component of the branchial arches in Akap95GT (left) and Figntm1Frk (right) animals. Labels are as follows: ma, mandibular component of branchial arch; mx, maxillary component of branchial arch.

Figure 8. Cleft palate phenotype in mice deficient in both fidgetin and AKAP95. A. Palatal views of a double homozygote and a littermate control. B. Palatal views of cleared skeletal preparations of a double homozygote and a littermate control. White arrows denote the boundary of the cleft and the vomer (star) is visible in the mutant. Scale bar: 1mm.
Table 1. Expression analysis of HA95 through real-time RT-PCR. PCR amplicons were designed to span introns and two non-overlapping primer pairs were used. Delta delta C_T, difference in threshold cycle value for mutant from the sex-matched wildtype littermate, shown is the average of three independent pairs; s.d., standard deviation. All C_T values were standardized against the loading control, actin mRNA.

|                  | Delta delta C_T | s.d. | % of control |                  | Delta delta C_T | s.d. | % of control |
|------------------|-----------------|------|--------------|-----------------|-----------------|------|--------------|
| 95gt/95gt        | 0.07            | 0.143| 95.26        | -0.082          | 0.078           | 105.7|              |
Table 2. Increased incidence of cleft palate in double mutant mice.

| Genotype         | # of mice analyzed | # of cleft palate | Percentage of cleft palate |
|------------------|--------------------|-------------------|----------------------------|
| $95^{th}/95^{th}$, $fi/fi$ | 34                 | 17                | 50                         |
| $95^{th}/+, fi/fi$ | 31                 | 6                 | 19.4                       |
| $+/+, fi/fi$      | 37                 | 7                 | 18.9                       |
| $95^{th}/95^{th}$, $fi/+ $  | 50                 | 1                 | 2                          |
| $95^{th}/95^{th}$, $+/>+$ | 20                 | 0                 | 0                          |
|          |  1  |  2  |  3  |  4  |  5  |  6  |  7  |  8  |
|----------|-----|-----|-----|-----|-----|-----|-----|-----|
| 95-FLAG  | +   | +   | +   | +   | +   | +   | +   |   |
| 95DelC-FLAG | +   | +   | +   | +   | +   | +   | +   |   |
| 95DelN-FLAG | +   | +   | +   | +   | +   | +   | +   |   |
| Fign-HA  | +   | +   | +   | +   | +   | +   | +   | +   |

**IP:** anti-FLAG

**Detect:** anti-HA

**Figure 3**
Figure 4

A.

U

Exon 8

D

Exon 9

Chr. 17

En2 \( \beta \)-geo pA

TD

B.

+/
het.
hom.
het.
DNA Marker

1,353 nt
1,078 nt
872 nt
603 nt
Figure 5

| hom. | het. | +/+ |
|------|------|-----|

- **AKAP95**
- **β tubulin**

1  2  3
Figure 6

gene trap fusion protein

AKAP95

Cy Nu Cy Nu
Figure 8

A. Control (palatal view)  Double mutant (palatal view)

B. Control (skeletal view)  Double mutant (skeletal view)
Interaction between fidgetin and protein kinase A-anchoring protein AKAP95 is critical for palatogenesis in the mouse
Yan Yang, Connie L. Mahaffey, Nathalie Bérubé and Wayne N. Frankel

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