

Frequent β -Catenin Mutations in Juvenile Nasopharyngeal Angiofibromas

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Juvenile nasopharyngeal angiofibromas (JNAs) are locally aggressive vascular tumors occurring predominantly in adolescent males. The pathogenesis of JNAs is unknown. Recently, JNAs have been reported to occur at increased frequency among patients with familial adenomatous polyposis, suggesting that alterations of the adenomatous polyposis coli (APC)/ β -catenin pathway might also be involved in the pathogenesis of sporadic JNAs. We analyzed somatic β -catenin and APC gene mutations in 16 sporadic JNAs from nonfamilial adenomatous polyposis patients using immunohistochemistry for β -catenin, and direct DNA sequencing for exon 3 of the β -catenin gene and the mutation cluster region of the APC gene. Nuclear accumulation of β -catenin was diffusely present in the stromal cells but not in the endothelial cells of all 16 JNAs. Activating β -catenin gene mutations were present in 75% (12 of 16) of JNAs. Six JNA patients also had recurrent tumors after surgery, and in all cases the β -catenin gene status of the recurrent JNA was identical to the initial tumor. No mutations in the mutation cluster region of the APC gene were detected in the four JNAs without β -catenin mutations. The high frequency of β -catenin mutations in sporadic JNAs and the presence of identical β -catenin gene mutations in recurrent tumors indicates that activating β -catenin gene mutations are important in the pathogenesis of JNAs. The immunohistochemical localization of β -catenin only to the nuclei of stromal cells further suggests that the stromal cells, rather than endothelial cells, are the neoplastic cells of JNAs. (*Am J Pathol* 2001, 158:1073–1078)

Juvenile nasopharyngeal angiofibroma (JNA) is an uncommon vascular tumor occurring almost exclusively in adolescent males. JNAs constitute ~0.5% of head and neck tumors.¹ Although histologically benign, they have been reported to recur after surgical therapy in 21 to 34% of patients and can be locally aggressive.^{1,2} JNAs typically arise from the posterolateral wall of the nasal cavity and grow by erosion of bone and displacement of adja-

cent structures; eventually these lesions can involve the nasopharynx, paranasal sinuses, orbit, and skull base with intracranial extension.¹ Histopathologically, JNAs are characterized by proliferating, irregular vascular channels within a fibrous stroma. The stromal compartment consists of plump cells that can be spindle or stellate in shape and give rise to varying amounts of collagen fibers.³

The pathogenesis of JNAs is unknown. However, a causal association between JNAs and familial adenomatous polyposis (FAP) has recently been suggested, with JNAs occurring 25 times more frequently in patients with FAP than in an age-matched population.^{4,5} FAP is an autosomal-dominant condition characterized by upper and lower gastrointestinal tract adenomas, a high tendency for the development of colorectal and periampullary adenocarcinomas, and by various extraintestinal manifestations including benign epidermal cysts, osteomas, desmoid tumors, and malignant tumors of the thyroid, liver, biliary tree, and brain.^{6–11}

FAP results from germline mutations in the adenomatous polyposis coli (APC) gene on chromosome 5q. The APC gene product regulates the level of β -catenin protein, which acts both as a submembranous component in cadherin-mediated cell-cell adhesion and as a downstream transcriptional activator in the Wnt signaling pathway.^{12,13} APC tumor suppressor protein, along with glycogen synthase kinase-3 β (GSK-3 β), promotes phosphorylation of serine/threonine residues encoded in exon 3 of the β -catenin gene.^{12,14,15} Phosphorylation is then followed by ubiquitin-mediated degradation of β -catenin protein.^{16,17} Loss of β -catenin regulatory activity resulting in accumulation of β -catenin protein can occur either by truncating APC gene mutations or by stabilizing β -catenin gene mutations at GSK-3 β phosphorylation sites.^{15,18,19} A majority of colorectal adenomas and carcinomas can be shown to contain either bi-allelic inactivation of the APC gene or activating β -catenin gene mutations.^{20,21}

The increased incidence of JNA in patients with FAP raised the possibility that either APC or β -catenin gene mutations affecting the APC/ β -catenin pathway might also be involved in the pathogenesis of sporadic JNAs. We therefore analyzed a series of JNAs from patients

Accepted for publication November 29, 2000.

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without FAP for mutations in both the *APC* and β -*catenin* genes.

Materials and Methods

Case Selection

The study population consisted of 16 primary JNAs and six recurrent JNAs from 16 patients who underwent surgical resection at The Johns Hopkins Hospital between 1984 and 1999. Tumor recurrences were resected from 4 months to 5 years after the original surgery (mean, 24 months). All patients were male and ranged in age from 10 to 24 years (mean, 14 years) at the time of initial surgery. None of the patients was known to have FAP and none was enrolled in The Johns Hopkins Polyposis Registry.

Immunohistochemistry for β -Catenin

Immunoperoxidase stain using diaminobenzidine as the chromogen was performed on the Techmate 1000 automatic staining system (BioTek Solutions, Tucson, AZ). Deparaffinized sections of formalin-fixed tissue were stained with β -*catenin* antibody (mouse monoclonal antibody; Becton Dickinson Transduction Laboratories, Lexington, KY) at 1:500 dilution after heat-induced antigen retrieval.²²

DNA Extraction

Microdissection of JNAs from hematoxylin and eosin-stained slides for DNA extraction was performed from formalin-fixed, paraffin-embedded specimens. Genomic DNA was extracted as described previously.²³ Corresponding normal control DNA was extracted from non-neoplastic nasopharyngeal mucosa in 15 of 16 patients (normal tissue was not available in the remaining case).

Mutation Analysis of the β -Catenin Gene

Genomic DNA from each sample was amplified by polymerase chain reaction (PCR) using the primer pair: 5'-ATGGAAC-CAGACAGAGGGGC-3' and 5'-GCTACTTGTCTGAGT-GAAG-3'. These amplified a 200-bp fragment of exon 3 of the β -*catenin* gene that encompasses the region for GSK-3 β phosphorylation. PCR reaction was performed under standard conditions in a 50- μ l volume using PCR Master [containing 1.25 U of *Taq* polymerase in Brij 35, 0.005% (v/v), dATP, dCTP, dGTP, and dTTP at 0.2 mmol/L, 10 mmol/L Tris-HCl, 50 mmol/L KCl, and 1.5 mmol/L MgCl₂ (Boehringer Mannheim, Mannheim, Germany)] and 1 μ mol/L of both 5' and 3' oligonucleotides with 40 cycles (94°C for 1 minute, 58°C for 1 minute, and 72°C for 2 minutes). PCR products were treated with shrimp alkaline phosphatase and exonuclease I (Amersham, Buckinghamshire, UK) before sequencing. Treated PCR products were sequenced directly with SequiTherm EXCEL II DNA sequencing kit (Epicentre, Madison, WI) using the internal primers: 5'-AAAGCGGCTGTTAGTCACTFF-3' and 5'-GACTTGGGAGGTATCCACATCC-3'. Oligonucleotides were

end-labeled with (γ -³²P)-ATP (New England Nuclear-DuPont, Boston, MA) using T4 polynucleotide kinase (New England Biolabs, Beverly, MA). All mutations were verified in both sense and antisense directions. Base substitutions in codons 32, 33, and the second position of codon 34 were further confirmed by *Hinf*I restriction endonuclease (Promega, Madison, WI) assay. The 200-bp PCR product for β -*catenin* contains two *Hinf*I restriction endonuclease sites, which yield DNA fragments of 7 bp, 55 bp, and 138 bp after digestion of the wild-type allele. β -*catenin* mutations in codons 32 and 33 yield only 62-bp and 138-bp fragments after digestion because of ablation of the first *Hinf*I site. Mutations in the second position of codon 34 yield 55-bp and 145-bp fragments because of ablation of the other *Hinf*I site.

Mutation Analysis of the *APC* Gene

Mutation analysis of the *APC* gene was performed only on cases that did not show detectable β -*catenin* mutations. Four sets of oligonucleotide primers (A1: 5'-CAGACTTATTGTGA-GAAGA-3' and A2: 5'-CTCCTGAAGAAAATTCAACA-3' for codons 1260 to 1359; B1: 5'-AGGGTCTAGTTTATCTTCA-3' and B2: 5'-TCTGCTTGGTGGCATGGTTT-3' for codons 1339 to 1436; C1: 5'-GGCATTATAAGCCCCAGTGA-3' and C2: 5'-AAATGGCTCATCGAGGCTCA-3' for codons 1417 to 1516; D1: 5'-ACTCCAGATGGATTTTCTTG-3' and D2: 5'-GGCTG-GCTTTTTTGTCTTAC-3' for codons 1497 to 1596) were used

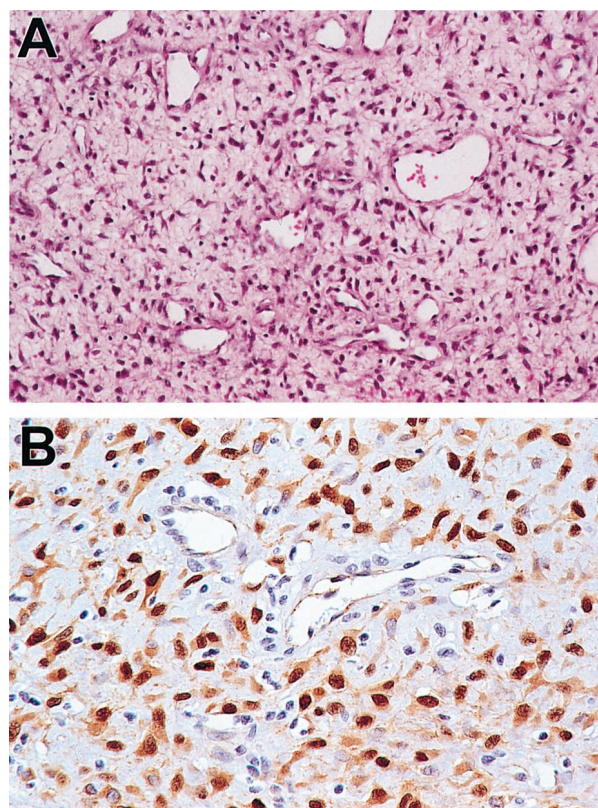


Figure 1. Histopathological appearance and immunohistochemical staining for β -*catenin* in JNAs. **A:** Tumors are comprised of spindle to stellate-shaped stromal cells and numerous vascular channels. **B:** Nuclear accumulation of β -*catenin* is diffusely present in stromal cells but not in endothelial cells or vascular smooth muscle cells, which show only faint cytoplasmic staining.

to amplify the mutation cluster region of the APC gene.²⁴ PCR reaction was performed under standard conditions in a 50- μ l volume using PCR Master (Boehringer Mannheim) and 1 μ mol/L of both 5' and 3' oligonucleotides with 40 cycles (94°C for 1 minute, 55°C for 1 minute, and 68°C for 2 minutes for APC-B, -C, and -D primer pairs and 94°C for 1 minute, 52°C for 1 minute, and 68°C for 2 minutes for APC-A). PCR products were purified using shrimp alkaline phosphatase and exonuclease I. Purified PCR products were sequenced directly with SequiTherm EXCEL II DNA sequencing kit using the same primers as for DNA amplification.

Results

Nuclear Accumulation of β -Catenin in JNAs

All 16 primary JNAs and the six recurrent tumors showed strong, diffuse nuclear staining for β -catenin (Figure 1). In all cases the nuclear staining was present only within stromal cell nuclei. Endothelial cell nuclei and the nuclei of vascular smooth muscle cells showed faint cytoplasmic staining but no nuclear β -catenin accumulation. Immunohistochemical staining of normal nasopharyngeal tissue was also evaluated in a total of 14 cases that included this tissue on the slides stained for evaluation of the JNAs (comprising 10 cases with surface epithelium,

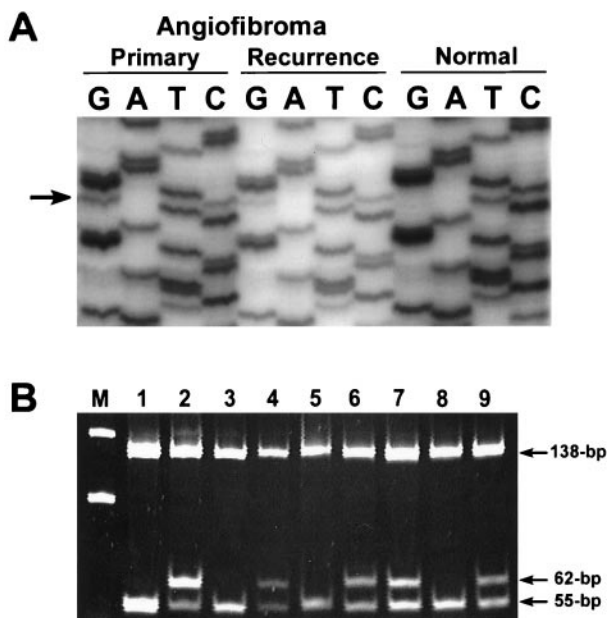


Figure 2. β -catenin gene mutations in JNAs. **A:** DNA sequencing autoradiograph of a TCT (serine)→TGT (cysteine) mutation (arrow) at codon 33, present in both the primary and recurrent JNA from patient 3. No β -catenin mutation is present in the corresponding normal nasopharyngeal mucosa from the same patient. **B:** *HinfI* restriction endonuclease assay to verify the presence of a point mutation in this case and in other representative cases with codon 33 mutations. DNA samples are from JNAs from patient 3 (lane 1, normal; lane 2, tumor), patient 6 (lane 3, normal; lane 4, tumor), patient 7 (lane 5, normal; lanes 6 and 7, primary and recurrent tumors), and patient 15 (lane 8, normal; lane 9, tumor). A molecular weight marker of 50-bp ladder is in lane M. The normal 200-bp PCR product for β -catenin contains two *HinfI* restriction endonuclease sites, yielding 7-bp, 55-bp, and 138-bp DNA fragments after digestion of the wild-type allele (the 7-bp fragment is too small to be visualized on the gel). β -catenin mutations in codon 33 yield 62-bp and 138-bp fragments after digestion because of ablation of the first *HinfI* site.

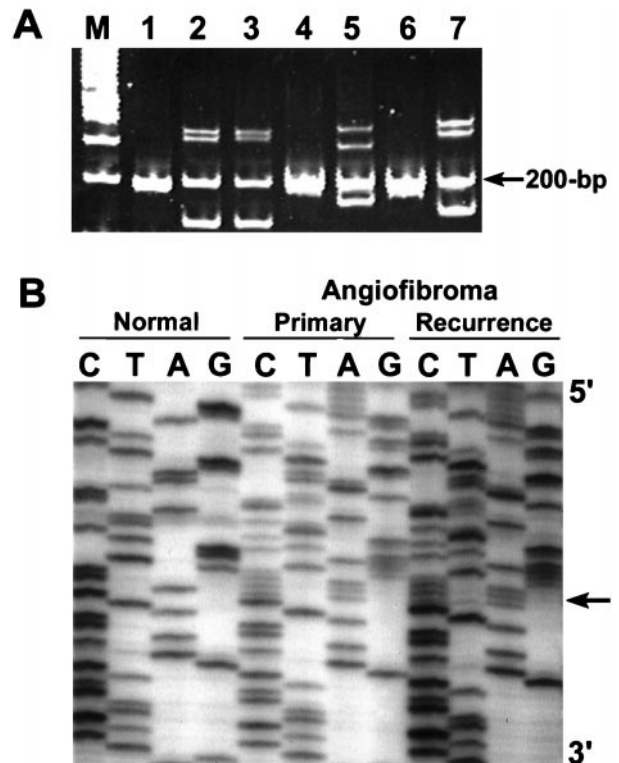


Figure 3. Deletion mutations in exon 3 of β -catenin in JNAs. **A:** Three JNAs with deletion mutations show β -catenin PCR products that are 164 bp, 182 bp, and 173 bp in addition to the normal 200-bp product (arrow). Patient 2 (lane 1, normal; lanes 2 and 3, primary and recurrent tumors); patient 8 (lane 4, normal; lane 5, tumor); and patient 11 (lane 6, normal; lane 7, tumor). Lane M contains a molecular weight marker of 50-bp ladder. **B:** DNA sequencing autoradiograph from patient 2, showing a 36-bp deletion beginning at codon 28 (arrow) in the primary and recurrent JNAs, but not in the normal nasopharyngeal mucosa from the same patient. Sequencing is shown in the 3' to 5' direction because of use of the reverse primer for sequencing.

eight cases with nonlesional fibrovascular stroma, seven cases with subepithelial lamina propria, and one case with cartilage). Strong membranous staining and light cytoplasmic staining for β -catenin was present within surface epithelium and seromucinous glands of the lamina propria in all cases that contained these elements. However, no nuclear accumulation of β -catenin was present in any nontumorous tissues.

Somatic β -Catenin Gene Mutations in JNAs

Mutations in exon 3 of the β -catenin gene were detected in 12 (75%) of 16 JNAs. The somatic nature of the mutations was confirmed by finding no β -catenin gene mutations in the corresponding normal nasopharyngeal tissue from these patients (available in 15 patients). In nine JNAs, mutations were 1-bp missense mutations, predominantly in one of the serine/threonine residues at GSK-3 β phosphorylation sites: codon 33 (five cases), codon 34 (two cases), codon 35 (one case), and codon 37 (one case) (Figure 2). Three additional JNAs contained deletions in exon 3 of β -catenin (an 18-bp deletion spanning codons 33 to 39, a 36-bp deletion spanning codons 28 to 40, and a 27-bp deletion spanning codons 39 to 48) (Figure 3). In all tumors with β -catenin gene mutations, a

Table 1. β -Catenin Gene Mutations in JNAs

Patient	Age (years)	JNA type	β -Catenin mutation	Nuclear accumulation of β -catenin protein
1	15	Primary	Codon 33, TCT \rightarrow GCT	+
2	24	Primary	Codons 28–40, 36-bp deletion	+
	29	Recurrent	Codons 28–40, 36-bp deletion	+
3	14	Primary	Codon 33, TCT \rightarrow TGT	+
	14	Recurrent	Codon 33, TCT \rightarrow TGT	+
4	12	Primary	–	+
	15	Recurrent	–	+
5	19	Primary	Codon 35, ATC \rightarrow AGC	+
	20	Recurrent	Codon 35, ATC \rightarrow AGC	+
6	12	Primary	Codon 33, TCT \rightarrow TGT	+
	14	Recurrent	Codon 33, TCT \rightarrow TGT	+
7	12	Primary	Codon 33, TCT \rightarrow TGT	+
	13	Recurrent	Codon 33, TCT \rightarrow TGT	+
8	12	Primary	Codons 33–39, 18-bp deletion	+
9	14	Primary	–	+
10	15	Primary	Codon 34, GGA \rightarrow CGA	+
11	10	Primary	Codons 39–48, 27-bp deletion	+
12	12	Primary	Codon 37, TCT \rightarrow TTT	+
13	10	Primary	–	+
14	16	Primary	–	+
15	13	Primary	Codon 33, TCT \rightarrow TGT	+
16	14	Primary	Codon 34, GGA \rightarrow CGA	+

mixture of wild-type and mutant bands was present, as would be expected because of the dominant-positive effect of β -catenin gene alterations. All five tumors with codon 33 mutations that could be evaluated with *HinfI* restriction endonuclease digestion showed the expected ablation of the *HinfI* recognition site.

Somatic APC Gene Mutations in JNAs

The four JNAs that did not show β -catenin gene mutations were further evaluated for APC mutations by sequencing of the APC mutation cluster region. However, no APC gene mutations were detected.

β -Catenin/APC Genes Mutations in Recurrent JNAs

Analysis of the six recurrent JNAs demonstrated identical genetic status between the primary tumor and recurrence. In five cases, identical β -catenin gene mutations were present in both primary and recurrent tumor, and in the remaining case no alteration in either β -catenin or APC was detected in the primary or recurrent tumor. A summary of genetic alterations in primary and recurrent JNAs is given in Table 1.

Discussion

The occurrence of nasopharyngeal angiofibromas in the nasal region of pubescent males, their histological similarity to erectile tissue, and their expression of multiple sex hormone receptors have remained tantalizing observations with possible implications about the origin of these neoplasms.^{25–27} However, little information concerning the molecular pathogenesis and specific neo-

plastic cell of origin of JNAs has previously been elucidated. We hypothesized that the increased frequency of JNAs among patients with FAP and germline mutations in the APC gene suggests that these tumors might arise through alterations of the APC/ β -catenin pathway.

We identified mutations in exon 3 of the β -catenin gene in 75% (12 of 16) sporadic JNAs from non-FAP patients. These mutations were predominantly 1-bp missense mutations in codons 33 and 37 (six cases total) leading to loss of serine/threonine sites for GSK-3 β phosphorylation. Other exon 3 mutations in codons 34 and 35 (three cases total) did not involve loss of a phosphorylation site but may still interfere with degradation of the β -catenin gene product.²¹ Three cases contained deletion mutations ranging from 18 bp to 36 bp and would be expected to interfere with β -catenin protein degradation. All six recurrent JNAs in our series showed β -catenin gene status that was identical to that of the corresponding primary tumors (β -catenin gene mutations in five cases and no β -catenin or APC mutation in the remaining case).

This high frequency of β -catenin gene mutations in sporadic JNAs, coupled with the increased frequency of JNAs among patients with FAP, suggests that these lesions arise through alterations of the APC/ β -catenin pathway. The presence of identical β -catenin gene mutations in the primary and recurrent tumors further supports that these genetic alterations are important and are maintained through the temporal growth of the tumors.

Somatic alterations of the APC/ β -catenin pathway have now been demonstrated in the sporadic counterparts to most tumors that occur frequently in FAP patients, including gastrointestinal adenomas,^{20,21,28,29} desmoid tumors,^{30–32} medulloblastomas,³³ childhood hepatoblastomas,^{34–35} and gastric fundic gland polyps.³⁶ β -catenin mutations have also been reported in a variety of other tumors such as anaplastic thyroid carcinoma,³⁷ prostatic

carcinoma,³⁸ endometrial carcinoma,^{39–41} pilomatricoma,⁴² Wilms' tumor,⁴³ and hepatocellular carcinoma.^{44–46} Among patients with FAP, intestinal and extra-intestinal neoplasms typically arise through bi-allelic (germline then somatic) inactivation of the *APC* gene, whereas the corresponding tumors in non-FAP patients occur either through somatic bi-allelic *APC* inactivation or somatic mutation of a single β -catenin allele. In contrast to the high frequency of β -catenin gene mutations detected in JNAs in this study, *APC* mutations in the mutation cluster region were not found. However, we did not sequence the entire *APC* gene in this study and we did not analyze tumors from patients with FAP, and therefore it remains possible that a specific subset of JNAs would contain bi-allelic inactivation of the *APC* gene.

The demonstration of nuclear β -catenin accumulation in all JNAs but β -catenin mutations in only 75% of the JNAs is interesting. Nuclear β -catenin accumulation can result from mutations in *APC*, β -catenin, or *AXIN1* (axis inhibitor 1) genes. The *AXIN1* gene encodes a key factor for the WNT signaling pathway, and interacts with *APC*, β -catenin, and GSK3 β , playing an important role in the degradation of β -catenin.⁴⁷ Somatic mutations of *AXIN1* have been reported in three of six hepatocellular carcinoma (HCC) cell lines and five of 87 primary HCCs.⁴⁸ In this study, we did not analyze for mutations in the *AXIN1* gene. However, the role of *AXIN1* in the pathogenesis of JNAs remains to be clarified.

All JNAs in our series showed strong immunohistochemical accumulation of β -catenin protein within the nuclei of stromal cells, whereas endothelial cells and vascular smooth muscle cells showed only faint cytoplasmic staining without nuclear β -catenin accumulation. JNAs are well known to be characterized histologically by proliferations of both vascular channels and spindled to stellate-shaped stromal cells that show ultrastructural features of fibroblasts,³ although the specific neoplastic cell has been debated. Some investigators have regarded JNAs as predominantly vasoproliferative malformations because of their tendency to bleed, their striking vascularity, and the presence of angiogenic growth factors within lesional endothelial cells.^{3,49} Schiff and colleagues⁴⁹ demonstrated intense immunohistochemical staining for the angiogenic protein basic fibroblast growth factor or FGF within all endothelial cells of JNAs, whereas only some stromal cells were stained. In contrast, Hwang and colleagues²⁷ found androgen receptor positivity by immunohistochemistry in both the endothelial cells and stromal cells of the majority of JNAs studied, although staining was typically more intense in the stromal cells. Our results indicate that JNAs are true neoplasms containing clonal alterations in the β -catenin oncogene, and suggest that the stromal component is the key neoplastic element of JNAs.

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