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Research article

Familial isolated pituitary adenoma is independent of *Ahr* genotype in a novel mouse model of disease

Anna L. Shen [a,*], Susan M. Moran [a], Edward N. Glover [a], Bernice C. Lin [a,b], Patrick R. Carney [a,c], Christopher A. Bradfield [a]

[a] *The McArdle Laboratory for Cancer Research, Department of Oncology, School of Medicine and Public Health, University of Wisconsin-Madison, Madison, WI, 53706, United States*
[b] *Current address, Lin-Zhi International, 2945, Oakmead Village Court, Santa Clara, CA, 95051, United States*
[c] *Department of Surgery, School of Medicine and Public Health, University of Wisconsin-Madison, Madison, WI, 53706, United States*

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ABSTRACT

Human familial isolated pituitary adenoma (FIPA) has been linked to germline heterozygous mutations in the gene encoding the aryl hydrocarbon receptor-interacting protein (AIP, also known as ARA9, XAP2, FKBP16, or FKBP37). To investigate the hypothesis that AIP is a pituitary adenoma tumor suppressor via its role in aryl hydrocarbon receptor (AHR) signaling, we have compared the pituitary phenotype of our global null *Aip* ($Aip^{\Delta C}$) mouse model with that of a conditional null *Aip* model ($Aip^{fx/fx}$) carrying the same deletion, as well as pituitary phenotypes of *Ahr* global null and *Arnt* conditional null animals. We demonstrate that germline $Aip^{\Delta C}$ heterozygosity results in a high incidence of pituitary tumors in both sexes, primarily somatotropinomas, at 16 months of age. Biallelic deletion of *Aip* in Pit-1 cells ($Aip^{fx/fx}$:*rGHRHRcre*) increased pituitary tumor incidence and also accelerated tumor progression, supporting a loss-of-function/loss-of-heterozygosity model of tumorigenesis. Tumor development exhibited sexual dimorphism in wildtype and $Aip^{fx/fx}$:rGHRHRcre animals. Despite the role of AHR as a tumor suppressor in other cancers, the observation that animals lacking AHR in all tissues, or ARNT in Pit-1 cells, do not develop somatotropinomas argues against the hypothesis that pituitary tumorigenesis in AIP-associated FIPA is related to decreased activities of either the *Ahr* or *Arnt* gene products.

1. Introduction

Familial Isolated Pituitary Adenoma (FIPA, OMIM 102200) is the familial occurrence of isolated pituitary adenomas in the absence of syndromic conditions and accounts for approximately 2% of clinically relevant human pituitary adenomas, prevalence of which has been estimated at 78–94 cases per 100,000 [1,2].Germline heterozygosity for loss-of-function mutations in the gene encoding a chaperone protein known as the aryl hydrocarbon receptor interacting protein (AIP, also known as ARA9, XAP-2, or FKBP37) have been reported in 10–30% of FIPA pedigrees, and up to 20% of sporadic pituitary adenomas [3–7], sparking interest in the role of AIP and its numerous interaction partners, including the aryl hydrocarbon receptor (AHR), in pituitary tumorigenesis. The AIP protein, a highly conserved 330 amino acid 38 kDa cytoplasmic protein containing an N-terminal FK506 binding protein (FKBP)-like

* Corresponding author.
E-mail address: alshen@wisc.edu (A.L. Shen).

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immunophilin domain and a C-terminal tetratricopeptide repeat (TPR) domain involved in protein-protein interactions [8,9], was first identified as a co-chaperone for the hepatitis B X protein [10] and the nuclear aryl hydrocarbon receptor (AHR) [11–13]. At least 20 interaction partners of AIP have since been identified [5,14–18] and involvement of numerous AIP-dependent signaling pathways have been proposed [5,14,15,18–21].

In humans, AIP-mutated pituitary adenomas are associated with younger age of onset relative to sporadic pituitary adenomas, preponderance of growth hormone (GH) or prolactin (PRL) secreting adenomas, and large and invasive tumors with poor response to somatostatin analog treatment [3,4,17,22,23]. The penetrance is low, approximately 20%, and males are more frequently affected [3,7,23–25]. Over 100 pituitary adenoma-associated human *AIP* variants have been identified, with over 70% leading to a truncated protein and the remaining 30% associated with disruption of the C-terminal TPR domain [5,26]. These observations, together with reports of absence of AIP protein in tumors, are evidence that AIP acts as a tumor suppressor in the pituitary, whereby a loss-of-heterozygosity (LOH) event in somatic cells results in inactivation of the functional wildtype *Aip* allele and complete loss of AIP function [27]. Supporting this model are studies demonstrating LOH in AIP-linked tumors [28] and in vitro studies demonstrating the ability of wildtype, but not mutant, AIP to decrease cell proliferation [29].

The essential role of AIP in the AHR signaling pathway is well-established [30,31] and tumor suppressor functions of AHR have been reported in a number of cancers [32–34]. Reports of decreased immunoreactivity in tumor tissue for AHR or its dimerization partner, aryl hydrocarbon receptor nuclear translocator (ARNT) [35,36], association of AHR with cell proliferation and cell cycle regulation in vitro [29,37], and association of AHR polymorphisms with pituitary tumors [38,39], have led to the idea that disruption of AHR signaling might play a role in AIP-associated pituitary tumorigenesis.

Mouse models of pituitary tumorigenesis have the potential to offer insights into pathogenesis of tumor development as well as tools for therapeutic testing. We and others have generated global *Aip* knockout models [40–42]. Global *Aip* knockout mice do not survive past mid-gestation [40] and reports of tumor development in *Aip* heterozygotes have been variable [41–43]. To investigate the potential for the AHR to play a role in FIPA, through its interactions with AIP, we established a two-phase plan. The first phase was to develop a model of *Aip*-dependent FIPA in the mouse, one that could add to a mechanistic understanding of the underlying biology through LOH studies and cell specific deletion ("conditional"). The second phase was to compare the *Aip*-dependent FIPA model, with the pituitary phenotypes of the *Ahr* and *Arnt* null mouse models. The underlying hypothesis of these experiments was that, if AHR signaling was linked to FIPA, its genetic deletion, or that of its cognate dimerization partner ARNT, should produce pituitary adenomas similar in cell specificity and histology to those induced by loss of AIP function.

We demonstrate that either germline *Aip* heterozygosity or biallelic deletion of *Aip* in cells of the Pit-1 lineage leads to development of pituitary adenomas with high penetrance at 16 months of age. Furthermore, we show that biallelic *Aip* deletion in the Pit-1 lineage accelerates tumor progression, consistent with the hypothesis that AIP acts as a tumor suppressor in the pituitary. Finally, we report that global homozygous deletion of *Ahr* or deletion of *Arnt* in cells of the Pit 1-lineage are not associated with pituitary tumor development. Taken in sum, these data are consistent with the idea that the role of AIP in pituitary tumorigenesis is independent of AHR signaling.

2. Methods

Animal studies were conducted according to a protocol (M005959) approved by the University of Wisconsin School of Medicine and Public Health (UW SMPH) Institutional Animal Care and Use Committee (IACUC). Mice were housed in a selective pathogen-free facility accredited by the Association for Assessment of Laboratory Animal Care (AALAC), on corncob bedding with chow (Mouse diet 9F 5020; PMI Nutrition International) and water *ad libitum*.

Gene targeting and genotyping. Generation, genotyping, and characterization of the $Aip^{\Delta Cfx}$(Aip^{tm1Bra}, hereafter referred to as $Aip^{\Delta C}$) global *Aip*-null and Aip^{fx} ($Aip^{tm2.1Bra}$) conditional *Aip*-null alleles have been described previously [31,40,44]. Animals carrying these alleles have been backcrossed to C57BL6/J in our laboratory for at least 10 generations for this study. In the $Aip^{\Delta C}$ line, exons 4 through 7 are inverted, deleting the C-terminal residues of the FKBP-like domain and all of the TPR domains [40]. Animals designated $Aip^{\Delta C/+}$ are heterozygous for the mutant $Aip^{\Delta Cfx}$ allele and are globally haploinsufficient for AIP [40]. The targeting strategy used to generate the Aip^{fx} line introduces *Lox-P* sites flanking exons 4 through 7, allowing Cre-recombinase-dependent deletion of the FKBP-like and TPR domains [44]. This line is maintained by breeding homozygous $Aip^{fx/fx}$ animals. Previous studies have shown that deletion of Exons 4 through 7 effectively removes the function(s) of AIP in embryonic development [40] and in maintaining levels of functional cytosolic AHR protein in the liver [44]. The AHR knockout line, (Ahr^{tm1Bra}, referred to as $Ahr^{\Delta 2}$), congenic on the C57BL6/J background, has been described previously [45]. This line is maintained by crossing heterozygotes ($Ahr^{\Delta 2/+}$) to generate heterozygous, null ($Ahr^{\Delta 2/\Delta 2}$), and wildtype progeny. The $Arnt^{fx}$ ($Arnt^{tm1Bra}$) conditional null allele has been described previously and is maintained by breeding homozygous $Arnt^{fx/fx}$ animals [46].

Tissue-specific Aip deletion. To generate the $Aip^{fx/fx}$*:rGHRHRcre* mouse, deleting both copies of *Aip* in pituitary somatotrophs, lactotrophs, and thyrotrophs (Pit-1 lineage), $Aip^{fx/fx}$ animals were crossed to rGHRHRcre mice which express Cre-recombinase under control of the rat growth hormone releasing factor receptor promoter [47]. These animals originated on the FVB/N background and have been backcrossed to C57BL6/J for 4 generations in our laboratory before use in these studies. Deletion of *Aip* in gonadotrophs, somatic cells of the gonads, adrenal cortex, spleen, and the ventromedial hypothalamic nucleus ($AIP^{fx/fx}$*:Sf1cre*) was accomplished by crossing $Aip^{fx/fx}$ animals to *Sf1cre* animals, which express Cre recombinase under control of the *Sf1* (also known as *Nr5a1*) promoter [48]. The Sf1-cre mice were backcrossed to C57BL6/J for 7 generations prior to these experiments [49]. Deletion of *Arnt* in Pit-1 cells ($Arnt^{fx/fx}$*:rGHRHRcre*) was accomplished by crossing $Arnt^{fx/fx}$ conditional null animals [46] to *rGHRHRcre* animals. In all crosses, Cre animals carried a single copy of the *Cre* allele, yielding *fx/fx* ($AIP^{fx/fx}$) unfloxed and *fx/fx-cre* ($Aip^{fx/fx}$*:rGHRHRcre* or $AIP^{fx/fx}$*:Sf1cre*)

Table 1
Mouse lines: Copy number and tissue-specificity.

Genotype	Copy Number (Germline)		Copy number (LOH)
	Global	Pituitary	Pituitary
Wildtype ($Aip^{+/+}$, $Ahr^{+/+}$)	2	2	1
[a] $Aip^{fx/fx}$	2	2	1
[b] $Aip^{\Delta C/+}$	1	1	0
[c] $Aip^{fx/fx}$:r*Ghrhr-Cre*	2	0 (Pit-1 lineage)	0 (Pit-1 lineage)
[d] $Aip^{fx/fx}$:*Sf1-Cre*	2	0 (gonadotrophs)	0 (gonadotrophs)
[e] $Ahr^{\Delta 2/\Delta 2}$	0	0	0
[f] $Arnt^{fx/fx}$	2	2	1
[g] $Arnt^{fx/fx}$:r*Ghrhr-Cre*	2	0 (Pit-1 lineage)	0 (Pit-1 lineage)

For each genotype, the expected number of *Aip*, *Ahr*, or *Arnt* wildtype alleles are presented. LOH copy number is the number of wildtype alleles expected after a loss-of-heterozygosity (LOH) event in the pituitary.
[a] Exons 4–7 of *Aip* flanked by LoxP sites for cell-specific deletion.
[b] Globally heterozygous for one *Aip* wildtype allele and one *Aip* mutant allele (deletion of Exons 4 through 7).
[c] Biallelic deletion of Exons 4–7 of *Aip* in cells of the Pit-1 lineage.
[d] Biallelic deletion of Exons 4–7 of *Aip* in gonadotropes.
[e] Global biallelic deletion of *Ahr* Exon 2.
[f] Exon 2 of *Ahr* flanked by loxP sites for cell-specific deletion.
[g] Biallelic deletion of *Arnt* Exon 6 in cells of the Pit-1 lineage.

excised progeny. Cre-mediated AIP excison was confirmed by PCR as described previously [44].

Pituitary analysis. Pituitary tumors were assessed at 16 months of age. For assessment of pituitary adenoma development, animals were euthanized with CO_2 and the sphenoid bone and sella turcica containing the pituitary was dissected. After 4 h of fixation in 10% phosphate-buffered formalin, pituitaries were dissected, dehydrated in a graded ethanol series, and embedded in paraffin on a Sakura Tissue-Tek VIP. The entire pituitary was sectioned at 10 μm and every 10th section stained with hematoxylin and eosin (H&E). Regressive H&E staining was done on a Leica Autostainer XL. In some cases, sections exhibiting evidence of tumors were also stained with reticulin and antibodies to growth hormone or prolactin. Reticulin staining was carried out as described [50]. Antibodies to mouse/rat growth hormone (anti-mrGH) and mouse prolactin (anti-mPRL) were purchased from NIDDK National Hormone and Peptide Program (A. F. Parlow). Samples were incubated in PBS with 1% goat serum at 4° overnight with antibody to growth hormone (1:1600) or prolactin (1:1600) followed by incubation with Signal Stain® Boost IHC Detection Reagent (HRP, Rabbit) (Cell Signaling Technologies) for 30 min at room temperature and incubation with diaminobenzidine (Cell Signaling Technologies) and counter-staining with Mayer hemotoxylin (Sigma). Sections were examined for the presence of tumors by three independent observers blinded to genotype. Tumors appeared as eosinophilic and hyperplastic areas upon H&E staining, with a disrupted acinar structure. Somatotropinomas were characterized by immunoreactivity to growth hormone antibody and lack of prolactin immunoreactivity. Prolactinomas exhibited prolactin, but not growth hormone, immunoreactivity. Tumors negative for prolactin and growth hormone immunoreactivity were classified as GH/PRL-negative tumors. Total tumors are defined as the sum of the three tumor types.

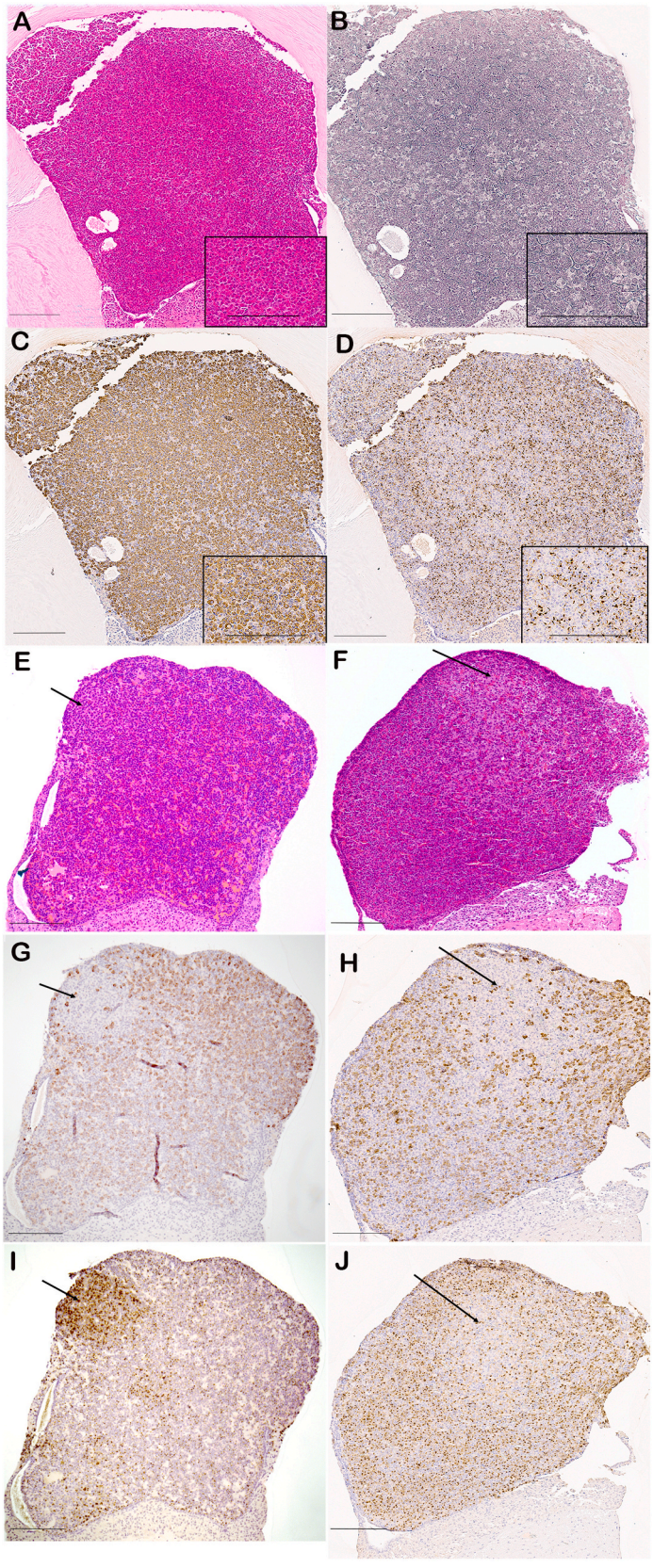
Statistical analysis. Statistical analysis was carried out using the software program Mstat, http://www.mcardle.wisc.edu/mstat/, or the statistical functions of Microsoft Excel. Prevalence was analyzed using Fisher's exact test. Tumor multiplicity is presented as mean ± standard deviation (number of animals) and significance of differences in tumor multiplicity was determined using the Kruskal-Wallis test. Significance of differences in body weight was determined using Student's *t*-test. For these analyses, $Aip^{\Delta C/+}$ animals were compared to $Aip^{+/+}$ sex-matched littermates and $Aip^{fx/fx}$:*rGHRHRcre and* $AIP^{fx/fx}$:*Sf1cre* animals with their respective $Aip^{fx/fx}$ sex-matched littermates. $Ahr^{\Delta 2/\Delta 2}$ and $Arnt^{fx/fx}$:*rGhrhrcre* animals were compared to age- and sex-matched wildtype $Aip^{+/+}$ animals.

3. Results

Generation of mouse knockout models. Table 1 lists gene copy numbers, globally and in the pituitary, of the recombinant models used in this study. The $Aip^{\Delta C/+}$ model is both a model of global haploinsufficiency, as well as a LOH model in which germline inactivation of one allele followed by somatic inactivation of the normal allele produces complete loss of AIP expression in individual cells. In contrast, cell-specific expression of Cre recombinase leads to biallelic, complete, loss of *Aip*, at a defined timepoint, in those cells in which Cre recombinase is activated from a transgenic promoter. Cre recombinase expression is detected in the anterior pituitary of *rGHRHRcre* animals as early as E13.5 and is localized specifically in cells of the *Pou1f1* (Pit-1) lineage [47]. $Aip^{fx/fx}$ animals crossed to *rGHRHRcre* mice ($Aip^{fx/fx}$:*rGHRHRcre*) animals excise *Aip* in cells of the Pit-1 lineage, including pituitary somatotrophs, lactotrophs, and thyrotrophs [47]. Fig. S1 demonstrates activity of cre recombinase in *rGHRHRcre* animals crossed to the Gt (ROSA)26Sor lacZ reporter line. $AIP^{fx/fx}$ animals crossed to *Sf1cre* mice lack Aip in pituitary gonadotrophs beginning at embryonic day 10.5 [48].

To examine the role of AHR signaling in pituitary tumorigenesis, we employed $Ahr^{\Delta 2/\Delta 2}$ animals, that lack AHR expression in all tissues [45,45], and $Arnt^{fx/fx}$ animals crossed to *rGHRHRcre* mice ($Arnt^{fx/fx}$:*rGHRHRcre*), in which ARNT is ablated in Pit-1 cells. *Ahr* and *Arnt* gene copy numbers for these models are shown in Table 1.

Pituitary adenomas in wild-type ($Aip^{+/+}$) and heterozygous ($Aip^{\Delta C/+}$) mice. Wildtype male pituitaries exhibit a well-defined


A
B
C
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E
F
G
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Fig. 1. Histology of wildtype pituitary, age 16 months. Scale bars are 200 μm. Arrows indicate tumors. Insets at the lower right of panels A–D present enlarged views. A, male H&E; B, male reticulin; C, male growth hormone IHC; D. male prolactin IHC; E and F, female H&E; G and H, female growth hormone IHC; I and J, female prolactin IHC.

Table 2
Pituitary tumor prevalence in 16-month-old *Aip* $^{+/+}$ and *Aip*$^{\Delta C/+}$ animals.

Genotype	Sex	n	Animals with No Tumors (%)	Animals with Tumors (%)			
				GH/PRL-negative	Somatotropinoma	Prolactinoma	Multiple tumor types
Aip$^{+/+}$ (wt)	M	11	91	9	0	0	0
Aip$^{\Delta C/+}$	M	15	7***	13	93***	27	40*
Aip$^{+/+}$ (wt)	F	11	27	55	0	45	50
Aip$^{\Delta C/+}$	F	13	8	62	77**	62	80

Tumor prevalence in 16-month-old *Aip*$^{\Delta C/+}$ animals. *P < 0.05 relative to wildtype male littermates. **P < 0.005 relative to wildtype female littermates. ***P < 0.0001 relative to wildtype male littermates.

Table 3
Body weights of AIP mouse models.

Genotype	Sex	Weight (g)
Aip$^{+/+}$	M	43.8 ± 8.8 (16)
Aip$^{\Delta C/+}$	M	44.9 ± 6.1 (20)
Aip$^{fx/fx}$ (for r*Ghrhr-Cre*)	M	45.9 ± 5.2 (13)
Aip$^{fx/fx}$:r*Ghrhr-Cre*	M	48.7 ± 6.0 (14)
Aip$^{fx/fx}$ (for *Sf1-Cre*)	M	35.7 ± 5.5 (10)
AIP$^{fx/fx}$:*Sf1-Cre*	M	28.8 ± 3.5 (8)*
Aip$^{+/+}$	F	35.4 ± 6.9 (14)
Aip$^{\Delta C/+}$	F	37.9 ± 8.5 (13)
Aip$^{fx/fx}$ (for r*Ghrhr-Cre*)	F	33.0 ± 5.6 (11)
Aip$^{fx/fx}$:r*Ghrhr-Cre*	F	41.3 ± 7.6 (11)*
Aip$^{fx/fx}$ (for *Sf1-Cre*)	F	30.7 ± 5.7 (9)
AIP$^{fx/fx}$:*Sf1-Cre*	F	28.6 ± 8.0 (6)

Mean body weights at 16 months of age. Values are shown as Mean ± SEM (n). * indicates P < 0.02 versus the corresponding *Aip*$^{fx/fx}$ control.

acinar structure by H&E (Fig. 1A) and reticulin staining (Fig. 1B). Immunohistochemistry reveals a relatively even distribution of cells reacting with antibodies to either growth hormone (Fig. 1C) or prolactin (Fig. 1D). Tumor prevalence in wildtype animals was sex-dependent. A single wildtype male developed a GH/PRL-negative tumor at 16 months of age. However, tumors were observed in 8 of 11 wildtype females (73%, Table 2), with a tumor multiplicity of 1.2 ± 1.1 tumors/animal. Tumors in wildtype females were visible as focal hyperplastic areas with a disrupted acinar structure upon H&E staining (Fig. 1E and F). These tumors were either prolactinomas, exhibiting prolactin immunoreactivity (Fig. 1I) with isolated growth-hormone expressing cells (Fig. 1G), or GH/PRL-negative tumors, largely negative for both growth hormone and prolactin immunoreactivity (Fig. 1, H and J). No somatotropinomas were observed in wildtype animals of either sex. Germline heterozygosity for the *Aip*$^{\Delta C}$ null allele was associated with an increase in tumor prevalence at 16 months of age in both males and females (Table 2), driven largely by increases in somatotropinomas. A 10-fold increase in total tumor prevalence was observed in *Aip*$^{\Delta C/+}$ males as compared to wildtype littermate controls (P < 0.0001, Table 2). Fourteen of fifteen 15 *Aip*$^{\Delta C/+}$ males developed somatotropinomas. Six animals developed a prolactinoma or GH/PRL-negative tumor in addition to a somatotropinoma. Although no somatotropinomas were observed in wildtype females, 77% of *Aip*$^{\Delta C/+}$ females developed somatotropinomas (P < 0.005, Table 2). Tumor development in *Aip*$^{\Delta C/+}$ animals was not accompanied by a significant increase in body weight (Table 3).

Tumors in *Aip*$^{\Delta C/+}$ male pituitaries are visible as focal hyperplastic lesions on H&E staining, (Fig. 2A), exhibiting an altered acinar structure and disrupted reticulin network (Fig. 2B). Tumors are isolated, with well-demarcated boundaries between normal and tumor tissue. Somatotropinomas are characterized by the presence of immunoreactivity to growth hormone (Fig. 2C) and absence of immunoreactivity to prolactin (Fig. 2D). Tumor multiplicity was 2.9 ± 1.7 (n = 14) tumors/animals. Although different tumor types were present in a single animal, tumors containing both growth hormone- and prolactin-expressing cells were not observed.

At 16 months of age, *Aip*$^{\Delta C/+}$ females developed multiple independent somatotropinomas in addition to prolactinomas and GH/PRL-negative tumors. Tumor multiplicity, 3.4 ± 2.4 (n = 13), was increased significantly (P = 0.009) relative to wildtype females. Unlike *Aip*$^{\Delta C/+}$ males, multiple cell types were observed within a single tumor. Fig. 2, E-H, presents views of a female pituitary, showing the presence of somatotropinomas and prolactinomas. A GH/PRL-negative tumor is visible as a focal hyperplastic lesion with a disrupted acinar structure upon H&E and reticulin staining (Fig. 2E and F), largely negative for growth-hormone and prolactin immunoreactivity, but containing isolated growth hormone-positive and prolactin positive cells (Fig. 2G and H).

Effect of cell-specific biallelic *Aip* deletion on pituitary tumor development. rGHRHRcre recombinase activity produces

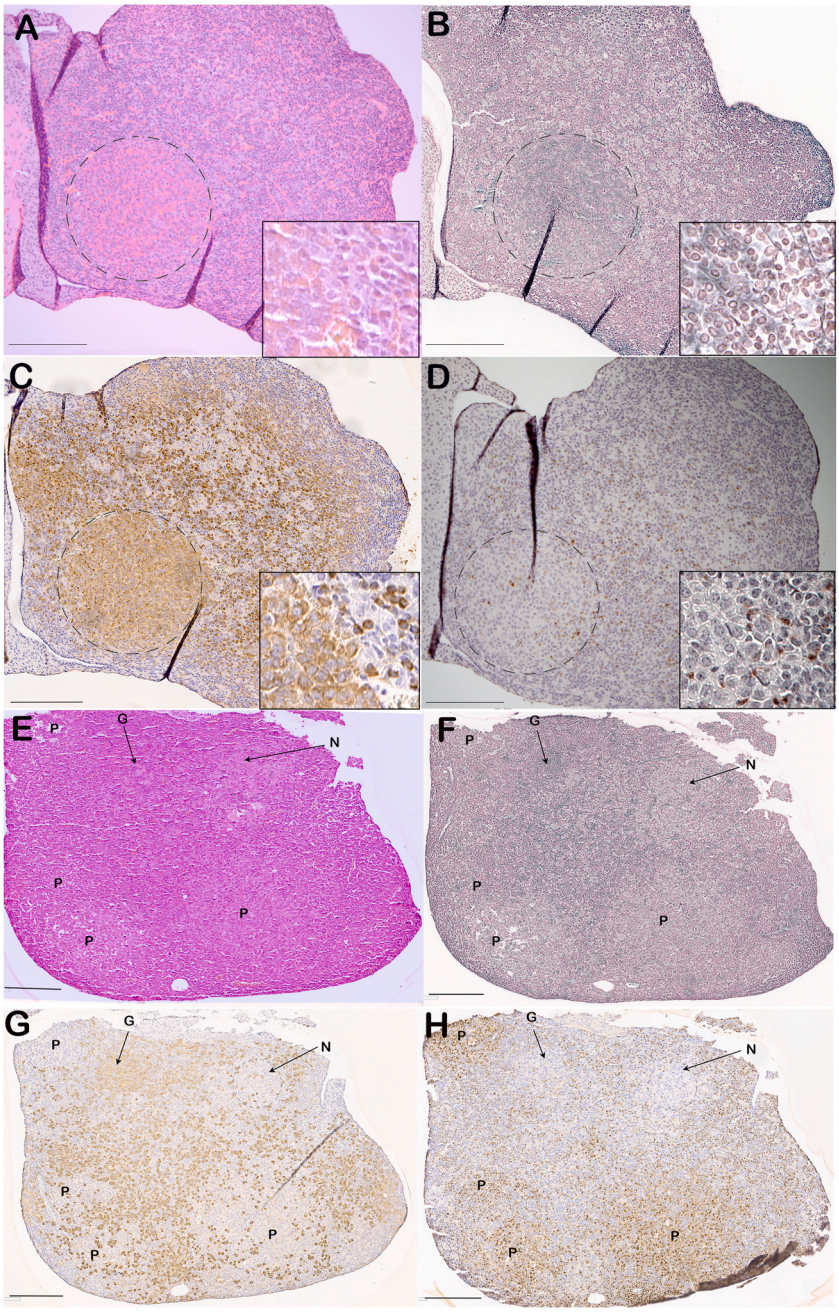

Fig. 2. Histology of $Aip^{\Delta C/+}$ pituitary, age 16 months. All scale bars are 200 μm. Males are shown in panels A–D. Dotted circles outline a somatotropinoma and insets show the demarcation between normal (right) and tumor (left) tissue. Females are shown in panels E–H. Arrows indicate tumors. Dotted circles outline a GH/PRL-negative tumor. G, somatotropinoma; P, prolactinoma; N, GH/PRL-negative tumor. A and E, H&E; B and F, reticulin; C and G, growth hormone IHC; and D and H, prolactin IHC.

biallelic *Aip* deletion at E13.5 in cells of the Pit-1 lineage [23]. $Aip^{fx/fx}$:*rGHRHRcre* males exhibited an increased growth rate, compared to $Aip^{fx/fx}$ males, between 2 and 4 months of age (Fig. 3A), suggestive of excess growth hormone secretion. However, after 4 months, body weights of $Aip^{fx/fx}$:*rGHRHRcre* and $Aip^{fx/fx}$ male controls were not significantly different (Fig. 3A and Table 3). $Aip^{fx/fx}$:*rGHRHRcre* females exhibited an increased rate of weight gain beginning at 2 months of age (Fig. 3, B), that persisted through 16 months of age (Fig. 3B, and Table 3).

As with global *Aip* heterozygosity, homozygous deletion of *Aip* in cells of the Pit-1 lineage also produced a marked increase in tumor prevalence in both sexes (Table 4). Tumors were observed in 93% of $Aip^{fx/fx}$:*rGHRHRcre* males ($P < 0.005$) and 94% of females ($P < 0.05$). Although increased tumor prevalence in $Aip^{fx/fx}$:*rGHRHRcre* males, like $Aip^{\Delta C/+}$ males, was due primarily to increased

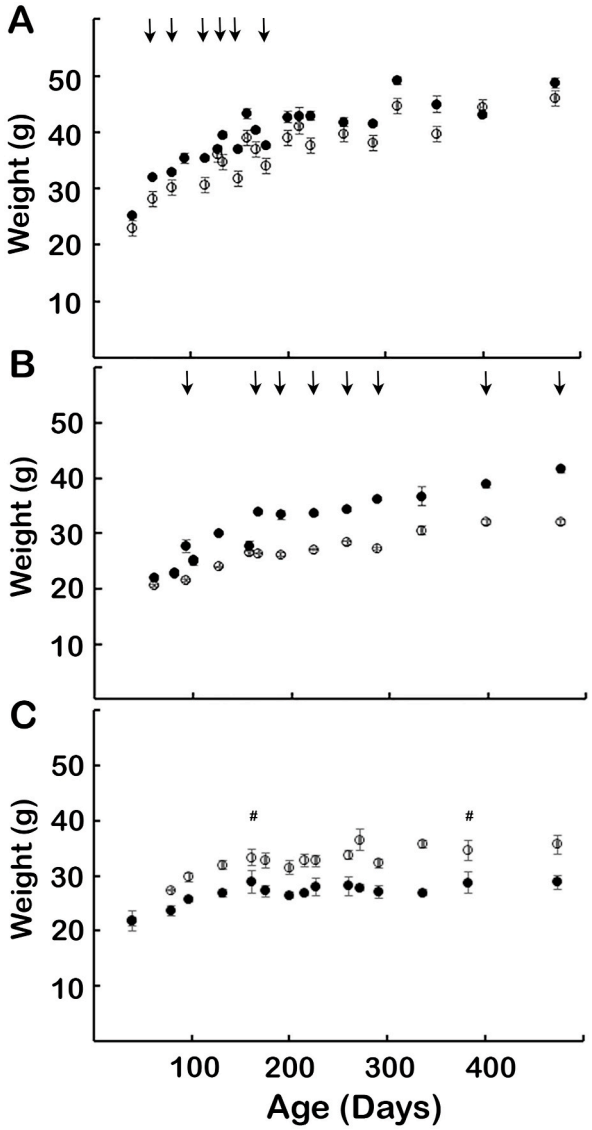

Fig. 3. Effects of tissue-specific Aip deletion on growth rate. Bars indicate SEM. A. Growth rate of *Aip*$^{fx/fx}$:r*GHRHR-Cre* males (closed circles) and *Aip*$^{fx/fx}$ male controls (open circles). Arrows indicate significant ($P < 0.05$) differences between *Aip*$^{fx/fx}$:r*GHRHR-Cre* and *Aip*$^{fx/fx}$. B. Growth rate of *Aip*$^{fx/fx}$:r*GHRHR-Cre* females (closed circles) and *Aip*$^{fx/fx}$ female controls (open circles). Arrows indicate significant ($P < 0.05$) differences between *Aip*$^{fx/fx}$:r*GHRHR-Cre* and *Aip*$^{fx/fx}$. C. Growth rate of *Aip*$^{fx/fx}$*:Sf1-Cre* males (closed circles) and *Aip*$^{fx/fx}$ male controls (open circles). Body weights at days indicated by # are not significantly different between *Aip*$^{fx/fx}$*:Sf1-Cre* and *Aip*$^{fx/fx}$. Body weights at all other time points are significantly different ($P < 0.05$) between *Aip*$^{fx/fx}$*:Sf1-Cre* and *Aip*$^{fx/fx}$.

somatotropinoma development (Table 4), AIP ablation in the lactotroph lineage produced an increase in prolactinoma prevalence.

Pituitary tumors in 16-month-old *Aip*$^{fx/fx}$*:rGHRHRcre* males exhibit evidence of increased tumor multiplicity and accelerated tumor progression. Unlike *Aip*$^{\Delta C/+}$ males, multiple, heterogeneous tumors (Fig. 4A–H) and grossly enlarged pituitaries (Fig. 4I–K) are observed in *Aip*$^{fx/fx}$*:rGHRHRcre* males. Although at least 15 tumors are visible in the pituitary shown in Fig. 4, panels A–D, lack of clear demarcation between tumors precluded an accurate determination of tumor multiplicity. Multiple tumor types are observed in a single pituitary (Fig. 4A–H). Reticulin fibers are largely absent. Hyperplastic cells exhibiting diffuse growth hormone immunoreactivity cover much of the pars distalis and demarcation between normal and tumor tissue is not apparent. GH/PRL-negative tumors, not seen in *Aip*$^{\Delta C/+}$ males, appear as eosinophilic areas on H&E that are largely negative for both PRL and GH, but contain isolated GH- and PRL-expressing cells. Chromophobic foci are present. Prolactinomas are visible as eosinophilic areas with positive PRL immunoreactivity and isolated GH-producing cells.

In contrast to *Aip*$^{fx/fx}$*:rGHRHRcre* males, tumors in *Aip*$^{fx/fx}$*:rGHRHRcre* females were generally isolated and well-demarcated, without the increased tumor multiplicity, tumor heterogeneity and increased pituitary size observed in *Aip*$^{fx/fx}$*:rGHRHRcre* males

Table 4
Tumor prevalence in 16-month-old animal models with cell-specific pituitary Aip deletion.

Genotype	Sex	n	Animals with No Tumors (%)	Animals with Tumors (%)			
				GH/PRL-negative	Somatotropinoma	Prolactinoma	Multiple tumor types
Aip$^{fx/fx}$	M	13	85	15	0	0	0
Aip$^{fx/fx}$*:rGHRHR-Cre*	M	14	7**	21	86**	36*	40*
Aip$^{fx/fx}$	F	11	55	9	18	64	20
Aip$^{fx/fx}$*:rGHRHR-Cre*	F	16	6*	38	50	88	70*
Aip$^{fx/fx}$	M	10	100	0	0	0	0
Aip$^{fx/fx}$*:Sf1-Cre*	M	8	62$^{\#}$	0	38$^{\#}$	12	10
Aip$^{fx/fx}$	F	11	36	27	10	55	30
Aip$^{fx/fx}$*:Sf1-Cre*	F	4	0	50	0	100	50

Tumor prevalence in 16-month-old animals. *P $<$ 0.05 relative to *Aip*$^{fx/fx}$ male or female littermates. **P $<$ 0.005 relative to *Aip*$^{fx/fx}$ male or female littermates. $^{\#}$P $<$ 0.07 relative to *Aip*$^{fx/fx}$ male littermates.

(Fig. 5A–C). The mean tumor multiplicity, 3.1 ± 1.7 (n = 16), was similar to that of *Aip*$^{\Delta C/+}$ females.

To investigate the effects of loss of AIP expression on tumorigenesis in other pituitary cell types, we deleted *Aip* in gonadotrophs. Homozygous deletion of *Aip* in Sf1-Cre expressing cells did not increase tumor prevalence in *Aip*$^{fx/fx}$*:Sf1-Cre* males compared to *Aip*$^{fx/fx}$ males (Table 4). However, males did exhibit significantly lower body weights and growth rates relative to *Aip*$^{fx/fx}$ littermates, suggesting derangement of the pituitary-gonadal axis. (Fig. 3C, and Table 3). Body weight and growth rates were not altered in *AIP*$^{fx/fx}$*:Sf1cre* females. Although tumor prevalence in *AIP*$^{fx/fx}$*:Sf1cre* females was not increased relative to *Aip*$^{fx/fx}$ (Table 4), prolactinoma multiplicity was increased from 0.8 to 2.8 tumors/animal (P = 0.02).

Lack of AHR signaling does not promote tumorigenesis. Examination of pituitaries from *Ahr*$^{\Delta 2/\Delta 2}$ animals at 16 months of age showed that tumor prevalence was not different from age- and sex-matched wildtype controls. GH/PRL-negative tumors were observed in 2 of 16 *Ahr*$^{\Delta 2/\Delta 2}$ males, a level not significantly different from that of age-matched wildtype males (Table 5). Prolactinoma prevalence in *Ahr*$^{\Delta 2/\Delta 2}$ females was not different from age-matched wildtype females (Table 5). No somatotropinomas were observed in any *Ahr*$^{\Delta 2/\Delta 2}$ animals. Tumor prevalence in *Arnt* $^{fx/fx}$*:rGHRHRcre* males and females, lacking *Arnt* expression in cells of the Pit-1 lineage, was also unchanged from that of wildtype animals. No somatotropinomas were observed in *Arnt* $^{fx/fx}$*:rGHRHRcre* animals (Table 5).

4. Discussion

Development of a novel model of *Aip*-mediated FIPA. Human germline mutations in the *AIP* gene have been linked to familial isolated pituitary adenoma, leading to the proposal that AIP acts as a tumor suppressor [20,51]. Haploinsufficiency models of tumor suppressor function postulate that decreased levels of a gene product (e.g. AIP) in germline heterozygotes carrying one functional (wildtype) and one inactive allele are sufficient for tumorigenesis [27]. In contrast, LOH models postulate that tumorigenesis in germline heterozygotes requires complete loss of *Aip* gene expression through a "second hit" inactivation of the wildtype allele in somatic cells [27]. In this study, we have compared these two models directly, using animals carrying identical deletions of AIP exons 4 through 7 in either the germline or in defined cell populations. Our heterozgous *Aip*$^{\Delta C/+}$ mouse, carrying one wildtype, functional allele and one mutant, inactive allele, is a model of global haploinsufficiency as well as LOH. In contrast *Aip*$^{fx/fx}$:r*GHRHRcre* and *Aip*$^{fx/fx}$*:Sf1-Cre* mice are homozygous null models that lack *Aip* expression, beginning in embryogenesis, in Pit-1 cells and gonadotrophs, respectively [48,52].

Our observations of essentially complete penetrance of pituitary adenomas in *Aip*$^{\Delta C/+}$ germline heterozygotes further confirms the role of AIP in pituitary tumorigenesis and are consistent with both LOH and haploinsufficiency models. Our observations of increased tumor multiplicity, increased growth rate, and accelerated tumor progression in *Aip*$^{fx/fx}$:r*GHRHRcre* relative to *Aip*$^{\Delta C/+}$ animals are consistent with a LOH model requiring complete inactivation of the *Aip* gene for pituitary tumorigenesis. As with human FIPA, the greatest effect of biallelic *Aip* inactivation is on somatotropinoma development [51].

Our mouse models display characteristics in common with human FIPA and with other mouse models of FIPA. The *Aip*$^{\Delta C/+}$ mouse is similar to heterozygous AIP deletion mouse models developed by others [41,42]. Raitila et al. [41] observed a high prevalence of pituitary adenomas at 12 months of age in male and female global heterozygotes of an *Aip* allele lacking exons 3 through 7, providing early evidence of the importance of AIP deficiency in tumors. Our model differs [41,42] in that it retains Exon 3, containing the bulk of the FKBP-like immunophilin domain, including residues Lys66-Lys69 that have been shown to make contacts with the client protein (AHR) [53]. The contribution of the FKBP-like domain to delayed onset is unknown. Although we did not observe any tumors at 12 months of age in *Aip*$^{\Delta C/+}$ animals, age of onset in the models of Raitala et al. and Kang et al. [42,43,54] has also been variable, suggesting that variable stability of the truncated proteins or the presence of unknown genetic or environmental factor(s) contribute(s) to tumor progression.

As in human FIPA, the largest effect of *Aip* deletion is on somatotropinoma development. However, tumor development occurs at a significantly later age than human FIPA. Although human FIPA is characterized by low penetrance and onset in childhood or early adulthood [51], mice heterozygous for AIP deletions exhibit full penetrance and late age of onset. It should be noted that all the mouse models delete the entire carboxy-terminal TPR domain that mediates protein-protein interactions. Although truncating mutations are found in 79% of human FIPA cases, examination of the location of these mutations shows that many are likely to retain partial function

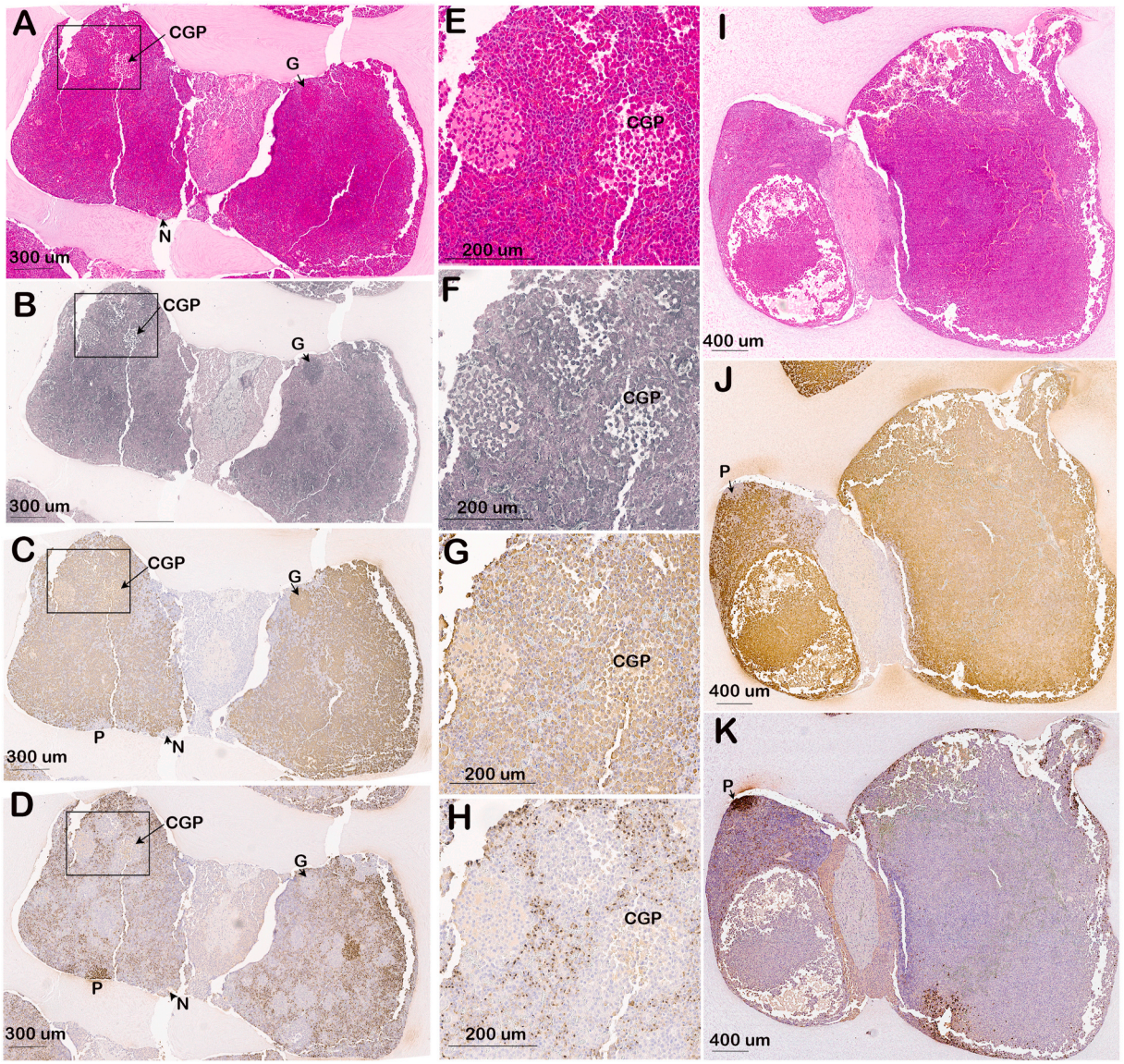

Fig. 4. Histology of *Aip*$^{fx/fx}$*:rGHRHRcre* male pituitaries, age 16 months, showing multiple heterogeneous tumors (A–H) or presence of large macroadenomas (I–K) in males. Scale bars are indicated on each panel. Panels E through H are higher magnification views of boxed areas in panels A through D. A, E, and I, H&E; B and F, reticulin; C, G, and J, growth hormone IHC; D, H, and K, prolactin IHC. Selected tumors are indicated. CGP, chromophobic and growth hormone- and prolactin-positive; G, growth hormone-positive; P, prolactin-positive; N, GH/PRL-negative.

that may contribute to decreased penetrance (https://www.ncbi.nlm.nih.gov/clinvar/?term=Aip%5Bgene%5D&redir=gene). Identification of the relevant AIP interaction partner and studies of the functional effects of individual mutations will be of benefit in establishing the presence of compensatory mechanisms contributing to tumor initiation and progression.

Models of cell specific deletion of *Aip* were developed to better understand the ontogeny of pituitary tumorigenesis, and to allow us to interrogate the role of alleles that are embryonic lethal when deleted globally (e.g., *Aip* and *Arnt*). The *Aip*$^{fx/fx}$*:GHRHRcre* mouse is comparable to the model employed by Gillam et al., where a Cre transgene under control of the rat growth hormone promoter (rGHpcretg/+: Aiplox/lox) was used to produce biallelic deletion of the TPR domain of the AIP protein [19] in somatotrophs beginning at approximately E17. Although both models eliminate the requirement for a loss-of-heterozygosity event, the GHRHRcre model targets other cells of the Pit 1 lineage in addition to somatotrophs leading to a spectrum of tumor types analogous to that observed in *Aip*$^{\Delta C/+}$ animals.

We observe a marked sexual dimorphism in tumor development in both *Aip*-sufficient (*Aip*$^{+/+}$ and *Aip*$^{fx/fx}$) and *Aip*-deficient (*Aip*$^{\Delta C/+}$ and *Aip*$^{fx/fx}$*:rGHRHRcre*) animals. Prolactinomas and non-expressing tumors developed in wildtype females but not wildtype males. Multiple cell types are found in tumors of wildtype and *Aip*$^{\Delta C/+}$ females, but not *Aip*$^{\Delta C/+}$ males. *Aip* heterozygosity increased somatotropinoma prevalence in *Aip*$^{\Delta C/+}$ animals of both sexes, in contrast to human FIPA which has been reported to occur more frequently in

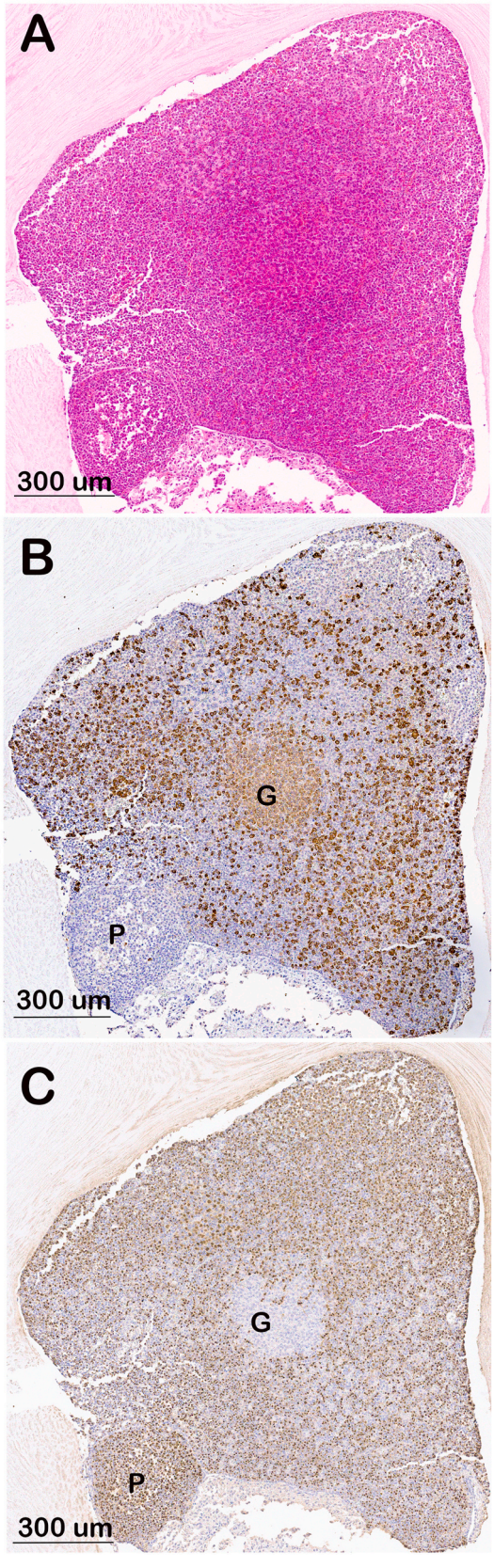

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Fig. 5. Histology of *Aip*$^{fx/fx}$*:rGHRHRcre* female pituitary, age 16 months. Scale bars are 300 μm. A, H&E; B, growth hormone IHC; and C, prolactin IHC. Representative growth hormone-positive and prolactin-positive tumors are indicated by G and P, respectively.

Table 5
Tumor prevalence in 16-month-old *Ahr*$^{\Delta 2/\Delta 2}$ and *Arnt*$^{fx/fx}$:r*GHRHRcre* animals.

Genotype	Sex	n	Animals with no tumors (%)	Animals with tumors (%)			
				GH/PRL-negative	Somatotropinoma	Prolactinoma	Multiple tumor types
wt	M	11	91	9	0	0	0
Ahr$^{\Delta 2/\Delta 2}$	M	15	87	13	0	0	0
wt	F	11	27	55	0	45	50
Ahr$^{\Delta 2/\Delta 2}$	F	6	67	0	0	33	0
Arnt$^{fx/fx}$:r*GHRHR-Cre*	M	4	100	0	0	0	0
Arnt$^{fx/fx}$:r*GHRHR-Cre*	F	4	25	75	0	25	33

Tumor prevalence in 16-month-old *Ahr*$^{\Delta 2/\Delta 2}$ and *Arnt* $^{fx/fx}$:r*GHRHR-Cre* animals. No significant differences between *Ahr*$^{\Delta 2/\Delta 2}$ animals and wildtype controls were observed.

males [23,25]. However, biallelic *Aip* deletion did have a greater effect in males. Despite evidence of increased growth hormone secretion in *Aip*$^{fx/fx}$:r*GHRHRcre* females, the increased somatotropinoma multiplicity and accelerated somatotropinoma development seen in *Aip*$^{fx/fx}$*:rGHRHRcre* males is not observed in *Aip*$^{fx/fx}$*:rGHRHRcre* females. We also note that accelerated prolactinoma development after gonadotrope *Aip* inactivation occurs in females but not males. These observations suggest a role for sex hormones in pituitary tumor promotion.

AIP-mutated FIPA is independent of AHR signaling. Early on, the AIP protein was identified as a chaperone for the hepatitis-B protein X and the AHR. More recently, additional AIP interaction partners have been implicated in pituitary tumorigenesis (reviewed in Barry and Korbonits [51]). The known function of AIP as a co-chaperone in AHR signaling has suggested a possible role for AHR and/or its heterodimerization partner ARNT in tumorigenesis. Reports that support such an idea include the observation that *Ahr* has been demonstrated to be a tumor suppressor gene in murine hepatocarcinogenesis [33]. Moreover, the rs2066853 *AHR* polymorphism has been associated with acromegaly and somatic loss of AHR Exon 10 has been reported in a cohort of human pituitary adenoma patients [38,39]. Observations of decreased ARNT in some human pituitary tumors [35] and lack of either ARNT or ARNT2 immunoreactivity in pituitary tumors [41] in *Aip* heterozygous mice, as well as reports of attenuated AHR signaling in human pituitary tumor samples [55] and fibroblasts from *Aip*-mutated patients [29] provided additional evidence for a role for AHR signaling in FIPA.

Given our expertise in the development of *Ahr, Arnt* and *Aip* recombinant mouse models, we set out to test the role of these loci in our AIP/FIPA model. While considerable data was in support of such an interaction, we also were aware of data that argued against this relationship. For example, decreased AHR signaling is a known consequence of AIP loss and we are not aware of a convincing causal link between altered AHR or ARNT signaling and pituitary tumorigenesis. Moreover, in our over twenty years working with these models we never observed phenotypes consistent with pituitary adenomas of any kind. Therefore, once we established a robust model of pituitary adenoma development due to *Aip* LOH, we turned our attention to the potential for LOF mutations at either *Ahr* or *Arnt* to also produce pituitary adenomas. Our observation that *Ahr*$^{\Delta 2/\Delta 2}$ and *Arnt*$^{fx/fx}$*:rGHRHRcre* animals do not develop pituitary tumors argues against the hypothesis that pituitary tumorigenesis is due to loss of AHR signaling and implicates the other AIP-dependent signaling pathway(s).

5. Ethics statement

Animal studies in this manuscript were approved by the Institutional Animal Care and Use Committee, University of Wisconsin School of Medicine and Public Health, Protocol #M005987-R01.

Data availability statement

All data associated with this study is provided in the manuscript. No data has been deposited in public repositories.

CRediT authorship contribution statement

Anna L. Shen: Writing – review & editing, Writing – original draft, Visualization, Investigation, Formal analysis. **Susan M. Moran:** Supervision, Project administration, Methodology, Investigation, Data curation, Conceptualization. **Edward N. Glover:** Methodology, Investigation, Conceptualization. **Bernice C. Lin:** Investigation, Conceptualization. **Patrick R. Carney:** Investigation, Formal analysis, Data curation. **Christopher A. Bradfield:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to

influence the work reported in this paper.

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Appendix A. Supplementary data

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