Comparative *PRKAR1A* genotype–phenotype analyses in humans with Carney complex and *prkar1a* haploinsufficient mice

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Carney complex (CNC) is a familial multiple neoplasia syndrome characterized by cardiac and extracardiac myxomas in the setting of spotty skin pigmentation and endocrinopathy. We previously identified *PRKAR1A* (regulatory subunit 1α of protein kinase A) mutations in CNC. Mutational analyses of the *PRKAR1A* gene in 51 unrelated CNC probands now detect mutations in 65%. All mutations, except for one unique missense mutation, lead to *PRKAR1A* haploinsufficiency. Therefore, we studied the consequences of *prkar1a* haploinsufficiency in mice. Although we did not observe cardiac myxomas or altered pigmentation in *prkar1a*$^{+/-}$ mice, we did observe some phenotypes similar to CNC, including altered heart rate variability. Moreover, *prkar1a*$^{+/-}$ mice exhibited a marked propensity for extracardiac tumorigenesis. They developed sarcomas and hepatocellular carcinomas. Sarcomas were frequently associated with myxomatous differentiation. Tumors from *prkar1a*$^{+/-}$ mice did not exhibit *prkar1a* loss of heterozygosity. Thus, we conclude that although PRKAR1A haploinsufficiency does predispose to tumorigenesis, distinct secondary genetic events are required for tumor formation.

In the autosomal dominant multiple neoplasia syndrome Carney complex (CNC, Online Mendelian Inheritance in Man 160980), recurrent heart, skin, and/or visceral myxomas are associated with spotty skin pigmentation, adrenal gland dysplasia, and schwannomas and pituitary, Sertoli, bone, ovarian, and breast tumors (1–6). Mutations in the chromosome 17q24 *PRKAR1A* gene encoding the R1α regulatory subunit of cAMP-dependent protein kinase A (PKA) cause CNC in some kindreds (7, 8). *PRKAR1A* mutant mRNAs were predicted to be degraded via nonsense-mediated decay (NMD), and *PRKAR1A* haploinsufficiency was hypothesized to cause CNC (7–9). Some, but not all, CNC tumors showed *PRKAR1A* loss of heterozygosity (LOH) and dysregulated PKA activity (8), but contributions of LOH and PKA activity to CNC tumorigenesis have remained controversial.

To assess contributions of *PRKAR1A* gene aberrations to CNC, we analyze here a cohort of 51 CNC probands. *PRKAR1A* mutations cause CNC in approximately two-thirds and, with one exception, an expressed missense mutation, produce *PRKAR1A* haploinsufficiency. Furthermore, we compared phenotypes of CNC patients to those of mice with *prkar1a* haploinsufficiency and identified physiologic abnormalities in both species. Our studies elucidate an expanded CNC phenotype in humans and mice with *PRKAR1A* haploinsufficiency. They demonstrate that *prkar1a*$^{+/-}$ mice are prone to tumor formation and further support that *PRKAR1A* haploinsufficiency is sufficient to foster CNC tumorigenesis. However, we also show that *prkar1a* LOH and altered tissue PKA activity are not required for murine or human tumorigenesis.

Methods

Human Clinical and Genetic Analyses. Informed consent was obtained as required by Weill Medical College's institutional review board, and participants were evaluated as described (10). Phlebotomy was performed, and lymphoblastoid cell lines were established (10). Genomic DNA was isolated, and genotyping and mutational analyses of *PRKAR1A* coding exons were performed by using denaturing HPLC and automated sequencing (7, 11). *PRKAR1A* cDNA was reverse-transcribed from RNA (RNeasy, Qiagen, Valencia, CA) with Superscript (Invitrogen) and PCR-amplified (30 s at 94°C, 30 s at 55°C, and 60 s at 72°C) $\times$ 35 cycles. Primers are available on request.

Analyses of *prkar1a*$^{+/-}$ Mice. *Prkar1a*$^{+/-}$ mice were generated by homologous recombination (12) and bred and studied per Cornell guidelines. M-mode and 2D echocardiography were performed

Freely available online through the PNAS open access option.

Abbreviations: CNC, Carney complex; NMD, nonsense-mediated decay; HRV, heart rate variability; PSC, premature stop codon; PKA, protein kinase A; LOH, loss of heterozygosity.

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with an Acuson (Mountain View, CA) Sequoia C256 and 15L8 probe. Mouse genomic DNA was purified from tail biopsies obtained under isofluorane anesthesia with ECG monitoring. To determine heart rate variability (HRV), 300 s of ECG data were acquired before biopsy and analyzed (13). Necropsy tissues were formalin-fixed and processed for microscopy (14). Rabbit polyclonal anti-R1α antibody (Chemicon) was used for immunohistochemistry with antigen retrieval and LSAB2 detection (Dako). Murine *prkar1a* cDNA was prepared by RT-PCR from TRIzol-isolated RNA (Invitrogen).

R1α Western Blot and PKA Activity Assays. Lysates from cultured cells or murine tissues were analyzed by Western blot with monoclonal anti-R1α antibody (BD Transduction Laboratories, San Diego; ref. 7). Recombinant R1α isoforms were expressed in COS-7 cells by Lipofectamine (BRL) transfection with pcDNA3.1 plasmids containing *PRKAR1A* cDNAs 3′ to cassettes encoding a FLAG tag (DYKDDDDK) and a linker (SGA). For Western blots, COS cell lysates were probed with anti-R1α and M2 anti-FLAG (Sigma). PKA activities were measured in triplicate in two independent laboratories (those of C.T.B. and G.S.M.) by using nonradioactive and radioactive PepTag assays (Promega). Protein kinase inhibitor (40 μg/ml; ICN) was used as a control inhibitor of PKA, and residual activity, if any, was considered background. For luciferase-based PKA assays, cells were plated in quadruplicate and cotransfected with *PRKAR1A* cDNAs and α168-luciferase plasmid (15), which contains a firefly luciferase reporter under the control of the human CG α promoter. Cells were also transfected with pRLTK (encoding *Renilla* luciferase) and pcDNA3.1-Myc/His-LacZ plasmids to control for transfection efficiency. Twenty-four hours after transfection, some cells were treated with 10^{-5} M forskolin (Sigma) and 0.5 mM 3-isobutyl-1-methylxanthine (Sigma) for 6 h and then assayed by Dual-Luciferase Reporter Assay (Promega). Statistical analyses were ANOVA (GB-Stat).

Results

***PRKAR1A* Genotypes in CNC.** Fifty-one unrelated CNC probands were evaluated. They represented 32 kindreds affected by autosomal dominant CNC and 19 apparently sporadic cases, i.e., no CNC family history. There were no significant differences in CNC manifestations between familial and sporadic cases. *PRKAR1A* was analyzed for mutations by both denaturing HPLC and sequencing in all probands. We identified *PRKAR1A* mutations in 20 of 32 familial and 13 of 19 sporadic probands (Table 1 and Fig. 4, which is published as supporting information on the PNAS web site). Thus, we observed *PRKAR1A* mutations in 65% (33/51) of CNC probands. Denaturing HPLC and sequence mutation scanning each identified 32 of 33 probands with *PRKAR1A* mutations. *PRKAR1A* mutations were identified among diverse ethnicities including Caucasian, Asian, Black, and Hispanic.

Of 33 probands with *PRKAR1A* mutations, 24 had unique mutations including four insertions, seven deletions, five nonsense mutations, eight splice site mutations, and one missense mutation, randomly distributed across the gene's exons. Two mutation hot spots were observed: Del(TG)576–577 (six probands) and C769T (four probands). Haplotype analyses with *D17S789* and *PRKAR1A-GA* (7) separated by 1.3 Mb confirmed that familial CNC probands sharing mutations were unrelated. The single missense mutation identified, C307T, predicts substitution of cysteine for arginine at residue 74 (R74C). R74 is highly evolutionarily conserved in humans, rodents, chick, frog, and zebrafish, and the mutant allele cosegregates with disease in family YPP ($Z = 1.5$ @ $\theta = 0$; Fig. 5, which is published as supporting information on the PNAS web site). No mutation was present in 100 unrelated normal chromosomes.

Clinical Features of Individuals with *PRKAR1A* Mutations. Probands with *PRKAR1A* mutations all had typical CNC features (Table 1) that did not significantly differ from those without *PRKAR1A* mutations. Eighty-five percent had either a personal or family history of cardiac myxomas, and 79% exhibited personal or family history of unusual skin pigmentation. Other CNC features included cutaneous myxomas, schwannomas, Sertoli cell tumors, trichofolliculoma, thyroid and pituitary adenomas, breast fibroadenomas, and adrenal hyperplasia. Some individuals exhibited clinical features not usually associated with CNC. Four of seven affected, but no unaffected, YC11 members had pilonidal sinuses. Three YZZ members had double ureter, spondylothesis, or tibia/femur fibromas. Congenital heart malformation occurred in four unrelated probands with *PRKAR1A* mutations. In two, cardiac outflow tract abnormalities [Tetralogy of Fallot (TOF) and pulmonic stenosis] were present. However, *PRKAR1A* mutations were not associated with outflow tract malformation outside CNC; analysis of 43 probands with TOF but without chromosome 22q deletions did not reveal *PRKAR1A* mutations. CNC phenotypes in YPP (R74C) were not unique and included spotty pigmentation, cardiac myxoma, thyroid adenoma, breast myxofibroma, and pulmonic stenosis. One affected member had congenital unilateral deafness.

Prior descriptions (16) hypothesized decreased CNC transmission by men vs. women. In our cohort, postadolescent women who have ongoing partners have had a mean of 2.9 children each, whereas men of reproductive age with ongoing partners have had only 1.6 offspring each. Degree of impaired male fertility is variable; offspring number for such CNC men ranges from 0 to 7 with a median of 1. Yet, 53% of these men have had children vs. 92% of these women. In addition, we observed abnormal semen analyses (oligospermia and/or abnormal sperm morphology) in five of five families (YA, YB, YJ, YT, and YZZ) carrying *PRKAR1A* mutations (K.B., unpublished work).

Expression Analyses of Mutant *PRKAR1A* Alleles. Previous studies have suggested that mutant *PRKAR1A* mRNAs with premature stop codons (PSCs) may undergo NMD (7–9) to produce *PRKAR1A* haploinsufficiency. Therefore, we sought to determine whether all *PRKAR1A* mutations we identified underwent NMD. Lymphoblastoid cell lines were derived from probands with the 33 *PRKAR1A* mutations. Sequence analysis of reverse-transcribed *PRKAR1A* cDNA revealed expression of WT *PRKAR1A* mRNA only for 29. When these 29 lines were treated with cycloheximide to inhibit NMD, both WT and mutant *PRKAR1A* alleles were observed (Fig. 6, which is published as supporting information on the PNAS web site). Thus, all these nonsense and frameshift mutations led to NMD.

PRKAR1A mutant mRNA from four lymphoblastoid lines carrying C997T, Del(A)1038, Δ-exon 7, or R74C mutations all escaped NMD, and sequence analysis of *PRKAR1A* cDNA from untreated cells indicated presence of both WT and mutant mRNA species for each. Unlike Del(A)1038, which occurs in the penultimate exon but introduces a PSC in the final exon, C997T occurs in the penultimate exon and introduces a PSC there; previous studies (17) demonstrated that boundaries of NMD surveillance mechanisms occur in penultimate exons. Neither Δ-exon 7 (in-frame deletion) nor R74C introduces PSCs, and thus both should evade NMD.

To determine whether these mutant mRNAs were translated to protein, we analyzed lymphoblastoid lines heterozygous for the C997T, Del(A)1038, Δ-exon 7, or R74C *PRKAR1A* mutations. Lysates from these lymphoblastoid lines were compared to WT lymphoblasts and lymphoblasts with haploinsufficient mutations leading to *PRKAR1A* NMD (Fig. 1*A*). Like other *PRKAR1A* haploinsufficient lymphoblasts, C997T, Del(A)1038, and Δ-exon 7 *PRKAR1A* lymphoblasts all exhibited ≈50% reduction in WT R1α protein without truncated R1α. Western blots of COS cells transfected with FLAG-tagged *PRKAR1A* cDNA constructs carrying the four mutations confirmed antibody reactivity with all mutant proteins (data not shown). By contrast, analysis (Fig. 1*A*) of

Table 1. CNC kindreds/probands with *PRKAR1A* mutations: Genotypes and clinical manifestations of disease in family members

Family	Country	*PRKAR1A* mutation	Mutation consequence	Cardiac myxoma	Skin myxoma	Lent.	Endocr.	Other tumors	CHD	Other phenotypes
YA	USA	Del(G)710	Fs-PSC	+	+	+	+	Schwannoma, TA, breast FA		
YB	USA	Del(TC)845–846	Fs-PSC	+	+	+	+	Breast FA, TA		
YC11	Ireland	Del(TG)139–140	Fs-PSC	−	+	+	−	Breast FA, gastric carcinoma		Pilonidal sinus
YD	USA	C584T	Nonsense	+	+	+	+			
YF	USA	Del(TG)576–577	Fs-PSC	+	−	+				
YJ	Belgium	Ins(GG)675	Fs-PSC	+	−	+	+	Sertoli, schwannoma		
YN	Ireland	Del(AA)745–746	Fs-PSC	+	+	+	+	Sertoli, schwannoma, breast FA, pituitary adenoma		
YO	Switzerland	C376T	Nonsense	+	−	−	+			
YP	Switzerland	Del(TG)576–577	Fs-PSC	+	+	+	+	Breast FA, TA		
YR	USA	Ins(C)632	Fs-PSC	+	−	+	−		ASD	
YT	USA	Del(T)706	Fs-PSC	+	+	+	−			
YW	Germany	Del(TG)576–577	Fs-PSC	+	+	−	+	Breast FA	PFO	Sudden death
YX	Japan	Del(GGTCTA)-1G-642	Splicing/in-frame Δ-Exon 7; deletion*	+	−	+	−			
YY	USA	C769T	Nonsense	+	−	+	−	Breast FA		
YZ	USA	C769T	Nonsense	−	+	+	+	Schwannoma		
YDD	France	IVS4 +1G > A	Splicing/Fs-PSC	+	−	+	−			
YEE	USA	IVS5 +1G ins(T)	Splicing/Fs-PSC	+	−	+	−	Trichofolliculoma		
YFF	Hong Kong	IVS6 −17T > A	Splicing/Fs-PSC	−	−	+	+			
YII	Australia	Ins(A)710	Fs-PSC	+	−	+	−	Sertoli		
YMM	Austria	C769T	Nonsense	+	+	−	−	Sertoli, breast FA		
YNN	Japan	C769T	Nonsense	+	−	−	−			
YPP	England	C307T	Missense: R74C*,†	+	−	+	−	Breast myxofibroma, TA	PS	Deafness
YSS	USA	Del(TG)576–577	Fs-PSC	+	−	−	−			
YTT	Japan	C997T	Nonsense*	+	−	−	+	TA, colon polyps		
YUU	Brazil	Ins(T)891	Fs-PSC	+	−	−	+	Breast FA		
YZZ	Finland	Del(TG)576–577	Fs-PSC	+	+	+	−	Tibia & femur fibromas		
CCE	Canada	Del(A)1038	Fs-PSC*	+	−	+	−	Sertoli, breast FA		
CCN	USA	IVS4 − 1 A > G	Splicing/Fs-PSC	+	−	+	+	Adrenal adenomas, sertoli		Blaschko's lines
CCR	England	IVS5 + 3 A > C	Splicing/Fs-PSC	−	−	+	−	Myxoid neurofibroma		
CCS	USA	IVS3 + 1 G > C	Splicing/Fs-PSC	−	−	+	−	Schwannoma, sertoli		
CCV	USA	Del(GATT)615–618, Ins(TATGATCAATC)615	Fs-PSC	+	−	+	−	Nasal hamartoma		Ankylosing spondylitis
SY13	Turkey	IVS8 + 5 G > C	Splicing/Fs-PSC	+	−	+	+		TOF	
SY21	USA	Del(TG)576–577	Fs-PSC	+	−	+	+			

Fs-PSC, frameshift mutation with consequent PSC; Splicing, mutation that alters *PRKAR1A* mRNA splicing; TA, thyroid adenoma; breast FA, breast fibroadenoma; ASD, atrial septal defect; PFO, patent foramen ovale; PS, pulmonic stenosis; TOF, Tetralogy of Fallot; CHD, congenital heart defect; Lent., lentiginosis; Endocr., endocrinopathy.

**PRKAR1A* mutant mRNA expressed and not subject to NMD.

†Mutant R1α protein expressed.

lymphoblasts from a family YPP proband, heterozygous for R74C-*PRKAR1A*, demonstrated no alteration in R1α protein levels.

Human *PRKAR1A* Mutations and PKA Activity. Previous analyses of CNC adrenal tumor cells suggested PKA hyperresponsivity to cAMP (8). Therefore, we tested whether the same phenomenon existed in CNC lymphoblasts. We found no *PRKAR1A* mutation had any detectable effect on basal or cAMP-stimulated PKA activity. No significant differences in PKA activities were observed among lymphoblasts with *PRKAR1A* haploinsufficient mutations or R74C, and these activities were not different from WT lymphoblasts (Fig. 1*B*). We also studied basal and cAMP-stimulated activities of R74C-R1α in a sensitive luciferase-reporter PKA assay (15). R74C-R1α was indistinguishable from WT R1α (Fig. 7, which is published as supporting information on the PNAS web site). Thus, R74C mutation does not alter R1α's ability to inhibit PKA activity.

A Mouse Model of *prkar1a* Haploinsufficiency. To further assess consequences of *PRKAR1A* haploinsufficiency, we studied *prkar1a*$^{+/-}$ mice (12). Although *prkar1a*$^{-/-}$ mice die with embryonic defects, *prkar1a*$^{+/-}$ mice undergo normal embryogenesis and lack malformations (12, 18). They are viable with normal lifespans. Pigmentation is normal but they exhibit impaired male fertility (K.B., unpublished work) similar to CNC men. Adult *prkar1a*$^{+/-}$ mice (5–11 mo) underwent echocardiography but were not different from WT littermates (Table 2) and had no intracardiac masses.

Electrocardiography revealed no heart rate changes or conduction delay, but HRV was significantly ($P = 0.005$) depressed in 11 *prkar1a*$^{+/-}$ mice [SD of normal to normal intervals (SDNN) = 5.4 +/− 0.5 msec] compared with 10 age-matched WT littermates (SDNN = 8.5 +/− 0.9 msec). Because decreased HRV is associated with increased risk of human sudden death (19–21), and sudden death has been observed in CNC (22), we asked whether humans with *PRKAR1A* haploinsufficiency had altered HRV.

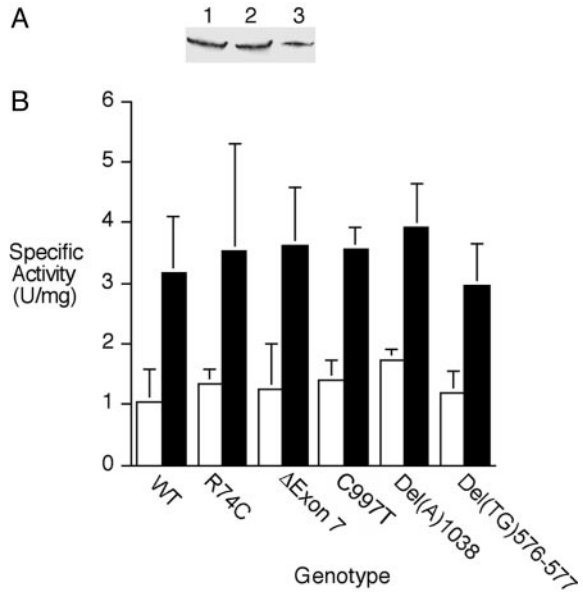

Fig. 1. R1α and PKA activity in CNC lymphoblasts. (*A*) Lymphoblast lysates analyzed by Western blot with anti-R1α. Lysate loads were normalized to equivalent amounts of β-actin. Shown are lymphoblasts from individuals who are unaffected (lane 1), from family YPP (R74C *PRKAR1A*) (lane 2), or from family YX (Δ-exon 7 *PRKAR1A*) (lane 3). Equal amounts of R1α are present in WT and YPP lymphoblasts, whereas R1α is reduced in YX lymphoblasts because of haploinsufficiency. No truncated R1α was detected. (*B*) Basal (open bars) and cAMP-stimulated (filled bars) PKA activities were measured for WT and CNC lymphoblasts heterozygous for different *PRKAR1A* mutations as indicated. Standard errors are shown. PKA activities do not differ among lymphoblasts with different *PRKAR1A* genotypes.

Twenty-four-hour ambulatory ECG recordings were available for analysis of six members (three adults and three children) of family YD (C584T *PRKAR1A* haploinsufficient mutation). We compared two affected adult siblings (ages 30 and 27) with an unaffected spouse (age 34) as well as an affected child (age 5) with two unaffected siblings (ages 4 and 7). Although no significant differences were noted between mean SDNNs, power spectral analysis revealed a loss of high-frequency variation in affected family members, and we calculated 30–60% reductions in affected individuals' rms of successful differences (rMSSD), an HRV measure largely influenced by high-frequency variation.

Tumorigenesis in *prkar1a*$^{+/-}$ Mice. *Prkar1a*$^{+/-}$ mice had increased frequency of extracardiac tumors. Necropsies were performed on 17 *prkar1a*$^{+/-}$ mice (9–19 mo; 10 males and 7 females), and 16 age-matched WT littermates (9 males and 7 females). Twelve of 17 *prkar1a*$^{+/-}$ mice had tumors (Fig. 2). There were no significant gender differences. Four *prkar1a*$^{+/-}$ mice had hemangiosarcomas of the spleen with myxoid regions. Splenic hemangiosarcomas were associated with metachronous lesions including colonic, ovarian, and pulmonary hemangiosarcomas. Two *prkar1a*$^{+/-}$ animals also had s.c. soft tissue myxoid fibrosarcomas of the forelimb. In addition, three other *prkar1a*$^{+/-}$ mice had soft tissue (spindle and chondro-) sarcomas with myxomatous differentiation invading the calvaria. Two mice had paraspinal and spinal sarcomas (chondro– and meningeal sarcomas) with cord compression and lower limb paralysis. Only one WT mouse had a tumor, a benign fibrous histiocytoma, which is common in older mice. Five *prkar1a*$^{+/-}$ mice had hepatocellular carcinomas. Two WT mice had hepatic foci of cellular alteration but with preserved architecture and without carcinoma.

Table 2. Echocardiographic analyses of *prkar1a* $^{+/-}$ and $^{+/+}$ mice

	prkar1a$^{+/-}$	*prkar1a*$^{+/+}$	*P* value
Heart rate, bpm	578 ± 51	545 ± 92	0.136
Septum, mm	0.46 ± 0.07	0.44 ± 0.16	0.708
LVIDD, mm	3.7 ± 0.47	3.61 ± 0.44	0.885
LVIDS, mm	1.78 ± 0.47	1.69 ± 0.26	0.964
LVPW, mm	0.57 ± 0.05	0.53 ± 0.12	0.852
Fractional shortening, %	51.8 ± 8.4	50.9 ± 5.2	0.802

bpm, Beats per min; LVIDD, left ventricular internal diameter in diastole; LVIDS, left ventricular internal diameter in systole; LVPW, left ventricular posterior wall.

Despite increased propensity to tumor formation and impaired male fertility, *prkar1a*$^{+/-}$ mice did not exhibit either *prkar1a* LOH or altered PKA activity (Fig. 3*A*). Two splenic hemangiosarcomas (Fig. 3*A*) and two soft tissue sarcomas (data not shown) all retained expression of WT R1α protein, and sequencing *prkar1a* cDNA revealed only WT mRNA without LOH. Immunohistochemistry of *prkar1a*$^{+/-}$ mouse tumors also consistently demonstrated persistent expression R1α protein in neoplastic cells (Fig. 3 *B–D*). Basal PKA activities in such tumors and their nontumor tissue counterparts were all barely detectable. Although cAMP-stimulated PKA activities were similar in WT and *prkar1a*$^{+/-}$ tissues (e.g., spleen and heart), enzymatic activities in tumors were highly variable (Fig. 3) and showed no consistent relationship to non-neoplastic counterparts.

Discussion

We identify *PRKAR1A* mutations in approximately two-thirds of CNC probands, higher than the 40% previously reported (9), and our studies of *prkar1a*$^{+/-}$ mice further support *PRKAR1A* mutations as important causes of CNC. Along with previous reports (7–9, 23), our data reveal two hot spots: Del(TG)576–577 in 21% of CNC probands with *PRKAR1A* mutations and C769T in 8%. Whereas most CNC patients have *PRKAR1A* mutations, a significant number do not. Indeed, for three of our probands without identified *PRKAR1A* mutations, their families had sufficient statistical power to test linkage to *PRKAR1A*, and we observed no linkage to chromosome 17q24 nor to a potential chromosome 2p16 locus (9). Thus, we observe marked CNC intergenic heterogeneity.

Although we discerned no unique phenotypes caused by specific mutations, we observed an expanded spectrum of clinical phenotypes in *PRKAR1A* mutations. Congenital heart malformations affecting the cardiac septa and outflow tract were variably present in humans with *PRKAR1A* mutations. A previous report (24) also noted right atrial myxomas in the setting of atrial septal defects. PKA activity has been implicated in specification of the cardiac conduction system (25) and in human hereditary cardiac arrhythmias and cardiomyopathies (26). Coupled with *cardia bifida* observed in *prkar1a*$^{-/-}$ murine embryos (12), our demonstration of an expanded CNC phenotype that includes congenital structural heart malformations extends the role of PKA signaling in human cardiogenesis.

Our findings also implicate *PRKAR1A* in extracardiac developmental processes. Fifty-seven percent of family YC11's affected members had pilonidal sinuses. Although these hair-containing sinuses in the sacrococcygeal region can represent acquired disease with hair follicle inflammation, leading to microabscess, Johnson-Villanueva (www.emedicine.com/emerg/topic771.htm) has suggested that pilonidal sinuses are medullary canal vestigial remnants or dermal inclusions caused by median coccygeal raphe maldevelopment. Association of pilonidal sinus with CNC and *PRKAR1A* mutation and a report of kindreds transmitting pilonidal sinus as an isolated autosomal dominant Mendelian trait suggest, at minimum, a hereditary predisposing abnormality if not an overt congenital defect.

Variable expressivity of CNC-causing haploinsufficient *PRKAR1A* mutations, which all reduce in R1α levels, is evident. For

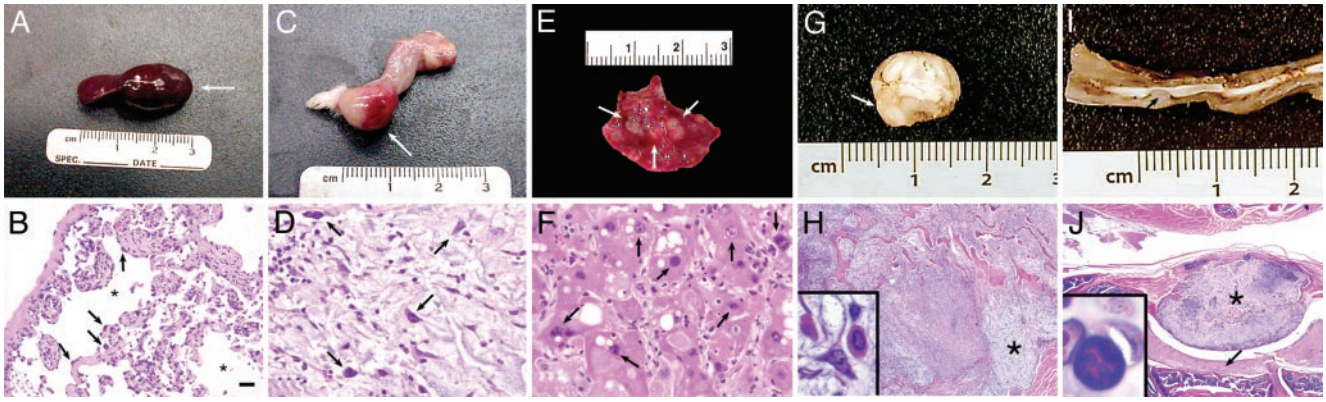

Fig. 2. Tumors in *prkar1a*$^{+/-}$ mice. Gross pathology (*A*, *C*, *E*, *G*, and *I*) and histology (*B*, *D*, *F*, *H*, and *J*) of tumors found in *prkar1a*$^{+/-}$ mice are shown. (*A* and *B*) The spleen was markedly enlarged by tumor (white arrow). Histology revealed splenic hemangiosarcoma with vascular channels lined by malignant endothelial cells (black arrows). (*C* and *D*) Left forelimb soft tissue mass (white arrow). Histology revealed myxoid fibrosarcoma comprised of highly anaplastic spindle cells (black arrows) with hyperchromatic enlarged nuclei and distinctive nucleoli dispersed in a myxoid stroma. (*E* and *F*) The liver (one lobe shown) had a multinodular (white arrows) appearance. Histology revealed hepatocellular carcinoma with multiple nodules comprised of malignant hepatocytes (black arrows) with loss of normal hepatic architecture. (*G* and *H*) Sarcoma mass involving the calvaria (white arrow) with distinct myxomatous areas (*). (*H Inset*) Typical stellate cell from myxomatous region. (*I* and *J*) Extradural lumbar chondrosarcoma causing compression and axonal degeneration in the spinal cord white matter (black arrow). (*J Inset*) A typical binucleated chondrocyte within the tumor. (Bar: 50 μm for *B*; 25 μm for *D* and *F*; and 10 μm for *H* and *J*.).

instance, in family YA [haploinsufficient Del(G)710 *PRKAR1A*], 9 of 16 affected individuals have cardiac myxomas, whereas 0 of 8 affected in family YC11 has had cardiac myxomas although they, too, have a haploinsufficient *PRKAR1A* mutation [Del(TG)139–140]. The failure to detect *PRKAR1A* genotype–phenotype correlations suggests that such phenotypic variability is likely a consequence of as-yet-unidentified modifying genetic and/or environmental factors. However, genotype–phenotype correlations may yet be found, and we are intrigued by *PRKAR1A* mutation clustering. Although the distribution of *PRKAR1A* mutations is random on an exon-by-exon basis, the distribution of mutations significantly deviates from random when considered on the basis of the four known R1α protein functional domains (dimerization, hinge, cAMP binding A, and cAMP binding B). Intriguingly, domain A exhibits a markedly greater predilection for mutation than the others ($P < 0.0001$) and includes nearly one-third of reported mutations (Fig. 4). Thus, it remains conceivable that rare mutant *PRKAR1A* mRNAs may evade NMD and have distinct pathologic consequences, or that other genetic and environmental factors may modulate NMD efficiency to modify CNC phenotypes. Future models of domain-specific R1α mutations will help address this issue.

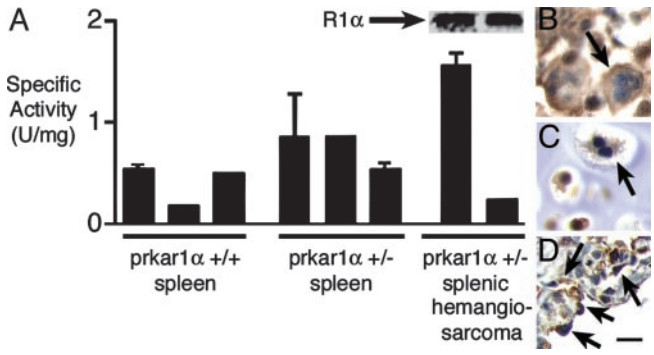

Fig. 3. PKA activity and R1α expression in *prkar1a*$^{+/-}$ murine tumors. (*A*) cAMP-stimulated PKA-specific activities of murine spleens and hemangiosarcomas. PKA assays were performed on freshly isolated WT (*prkar1a*$^{+/+}$) and *prkar1a*$^{+/-}$ spleens and splenic hemangiosarcomas from *prkar1a*$^{+/-}$ mice. cAMP-stimulated PKA activities are shown along with standard error. WT and *prkar1a*$^{+/-}$ spleens do not exhibit significantly different cAMP-stimulated PKA activities. cAMP-stimulated activities in splenic hemangiosarcomas are variable but are not consistently increased or decreased compared with those of *prkar1a*$^{+/-}$ spleens. (*Inset*) Western blot with anti-R1α of tissues shows expression of R1α in the tumors, i.e., absence of LOH. (*B–D*) Immunohistochemistry for R1α protein in *prkar1a*$^{+/-}$ murine tumors reveals the presence of staining (brown) for R1α in all tumor cells (arrows) and excludes *prkar1a* LOH. Shown are typical multinucleated tumor cells from a sarcoma (*B*), typical binucleated chondrosarcoma cells (*C*), and hyperchromatic anaplastic splenic hemangiosarcoma cells (*D*). (Bar: 25 μm.)

We demonstrate that four mutant *PRKAR1*α mRNAs [Δ-exon 7, R74C, Del(A)1038, and C997T] evade NMD. Δ-Exon 7 and R74C mRNAs would not be expected to undergo NMD because neither introduces a PSC. Previous studies (17) have proposed a 3′ boundary of 55 nt from the end of the penultimate exon as the NMD limit. Therefore, it is not surprising that Del(A)1038 *PRKAR1A* mRNA, containing a frameshift in the penultimate exon with a consequent PSC in the final exon, is not degraded. However, C997T introduces a PSC 63 nt before the penultimate exon's end and thereby extends the NMD surveillance 3′ boundary.

Despite NMD evasion, we do not detect R1α protein containing Δ-exon 7, Del(A)1038, or C997T mutations. Groussin *et al.* (23) described an in-frame exon 7 deletion and reported possible mutant protein expression. However, they did not demonstrate concomitant reduction in WT R1α that would be expected with *PRKAR1A* heterozygosity. Given the presence of stable mRNA for these alleles, we hypothesize that if any protein is encoded, it is misfolded and rapidly degraded in the endoplasmic reticulum (ER), thus not seen by Western blot. This model raises the possibility that low, undetected levels of mutant R1α may still be present and could have biologic effects, perhaps by activation of ER stress responses that have been implicated in other disorders, including hepatocellular carcinoma, Parkinson's disease, Alzheimer's disease, diabetes, cystic fibrosis, and autoimmunity (27).

By contrast, stable expression of R74C missense mutant R1α protein suggests that as-yet-unidentified mechanisms in addition to *PRKAR1A* haploinsufficiency may predispose to tumorigenesis. R74C occurs outside any known functional domain, and our data suggest that it can inhibit PKA activity just like WT R1α. Thus, we hypothesize that R74C can alter R1α higher-order structure and/or modify R1α interaction with partner proteins outside the PKA tetramer (e.g., A kinase anchoring proteins).

In this regard, similarities and dissimilarities between human CNC phenotypes and murine *prkar1a* haploinsufficiency are instructive. Two CNC hallmarks, spotty pigmentation and cardiac

myxomas, are not evident in *prkar1a*$^{+/-}$ mice. Similar discordance between human and murine skin findings occurs in *Lkb1*$^{+/-}$ mice that, unlike their human counterparts with Peutz–Jeghers syndrome, have normal pigmentation. However, as in humans, *Lkb1* haploinsufficiency does produce gastrointestinal polyps in mice. By contrast, absence of cardiac myxomas in *prkar1a*$^{+/-}$ mice suggests that either *prkar1α* haploinsufficiency is insufficient to produce cardiac tumorigenesis and/or that murine cardiac tissue has protective mechanisms not extant in humans. Both models suggest that additional genetic processes may synergize with *PRKAR1α* haploinsufficiency to produce variable CNC phenotypes.

Nonetheless, *prkar1a*$^{+/-}$ murine propensity for extracardiac tumors supports a role for *prkar1a* as a tumor suppressor. *prkar1a*$^{+/-}$ mice exhibit sarcomas, usually with myxomatous features. Sarcomas, like myxomas, are presumed to be mesenchymally derived "stromal" tumors based on the presence of markers such as vimentin, and some myxoid sarcomas and myxomas (e.g., intramuscular myxomas) that exhibit hypercellular regions are difficult to distinguish histologically (28, 29). Moreover, others have invoked a relationship of vascular proliferative lesions to myxomas because myxoma cells can express "endothelial" molecular markers (29–31). CNC's relationship to hepatocellular carcinoma is less clear; *PRKAR1α* was initially identified as a locus that repressed transcription specifically in hepatocellular carcinoma cells (32, 33). Disparities between *prkar1a*$^{+/-}$ mice and CNC may reflect interspecies differences in predilection for different tumor types; these are common, e.g., between human neurofibromatosis and *Nf1*$^{+/-}$ mice (34, 35).

Tumors that arise in *prkar1a*$^{+/-}$ mice do not display *prkar1a* LOH and human CNC tumors inconsistently exhibit *PRKAR1A* LOH. Previous reports observed preservation of the WT *PRKAR1A* allele in some human CNC tumors, including cardiac myxomas and pituitary adenomas (8, 36), and we demonstrate here that *prkar1a*$^{+/-}$ mouse tumors similarly preserve the WT allele. We do not observe significant differences in PKA activities between WT and *prkar1a*$^{+/-}$ murine tissues such as spleen and myocardium. Absence of cardiomyopathy in both *prkar1a*$^{+/-}$ mice and CNC patients as opposed to that seen in PKA catalytic subunit-overexpressing mice (39) also implies preservation of normal cardiac PKA activity. We did not observe altered PKA activity in *PRKAR1A* haploinsufficient human lymphoblasts. No consistent pattern of PKA activity alteration was observed in *prkar1a*$^{+/-}$ murine tumors. Although others have reported altered PKA activity in pituitary adenomas and lymphoblasts (36, 38), those activities are apparently not inhibited by protein kinase inhibitor and thus likely represent potentially nonspecific kinase activities not catalyzed by PKA. Our data do not exclude *PRKAR1A* haploinsufficiency-associated modification of PKA activity in cell types not evaluated here. In fact, altered HRV in *prkar1a*$^{+/-}$ mice and individuals with CNC suggests abnormal autonomic tone potentially mediated via altered central or peripheral nervous system PKA activity.

Our data, then, suggest that abnormalities in genes other than *PRKAR1α* are also required for tumorigenesis in both humans and mice. Such genetic events remain to be defined, but they could occur in as-yet-unidentified CNC genes. Additional candidate genes for CNC could include those encoding components of the PKA-signaling pathway (catalytic subunits, adenylate cyclases, and cAMP-phosphodiesterases), the numerous intracellular targets of these proteins, and A-kinase anchoring proteins (AKAPs). Few R1α-binding AKAPs have yet been identified, but include D-AKAP1, D-AKAP2, and myosin VIIa (39–41). Interestingly, the R1α domain that interacts with myosin VIIa (41) includes R74, which is mutated in family YPP; myosin VIIa mutations are associated with hereditary sensorineural deafness, which is also seen in family YPP. In addition, genes such as *LKB1*, *PTEN*, *PTCH*, and *PTPN11* associated with disorders (Peutz–Jeghers, Bannayan–Zonana, Gorlin, and LEOPARD syndromes, respectively) that share elements of the human CNC phenotype are intriguing targets for further exploration. Such studies will offer further insights into the pathogenesis of CNC and familial myxoma syndromes.

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