ORIGINAL ARTICLE

A transgenic mouse bearing an antisense construct of regulatory subunit type 1A of protein kinase A develops endocrine and other tumours: comparison with Carney complex and other *PRKAR1A* induced lesions

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Background: Inactivation of the human type Iα regulatory subunit (RIα) of cyclic AMP dependent protein kinase (PKA) (*PRKAR1A*) leads to altered kinase activity, primary pigmented nodular adrenocortical disease (PPNAD), and sporadic adrenal and other tumours.
Methods and results: A transgenic mouse carrying an antisense transgene for *Prkar1a* exon 2 (X2AS) under the control of a tetracycline responsive promoter (the *Tg(Prkar1a*x2as)1Stra*, *Tg(tTAhCMV)3Uh* or tTA/X2AS line) developed thyroid follicular hyperplasia and adenomas, adrenocortical hyperplasia and other features reminiscent of PPNAD, including late onset weight gain, visceral adiposity, and non-dexamethasone suppressible hypercorticosteronaemia, with histiocytic, epithelial hyperplasias, lymphomas, and other mesenchymal tumours. These lesions were associated with allelic losses of the mouse chromosome 11 *Prkar1a* locus, an increase in total type II PKA activity, and higher RIIβ protein levels; the latter biochemical and protein changes were also documented in Carney complex tumours associated with *PRKAR1A* inactivating mutations and chromosome 17 *PRKAR1A* locus changes.
Conclusion: We conclude that the tTA/X2AS mouse line with a downregulated *Prkar1a* gene replicates several of the findings in Carney complex patients and their affected tissues, supporting the role of RIα as a candidate tumour suppressor gene.

In the heterozygote state, *PRKAR1A* inactivating mutations cause adrenocortiotrophic hormone (ACTH; corticotrophin) independent Cushing's syndrome due to primary pigmented nodular adrenocortical disease (PPNAD) or Carney complex (CNC), a multiple neoplasia syndrome.[1–3] Somatic mutations of the *PRKAR1A* gene have been found in sporadic PPNAD and other adrenocortical tumours and, rarely, in non-medullary thyroid cancer.[4 5] In CNC associated tumours, and in adrenal and thyroid neoplasms with *PRKAR1A* down-regulation, allelic losses of the 17q22-24 *PRKAR1A* chromosomal locus are frequent and are associated with concomitant changes in cAMP stimulated protein kinase (PKA) activity.[1 4–6]

PKA is a serine–threonine kinase that mediates cAMP regulation for a variety of cellular processes.[7 8] The PKA regulatory subunits, *PRKAR1A* (RIα), *PRKAR1B* (RIβ), *PRKAR2A* (RIIα), and *PRKAR2B* (RIIβ), and catalytic subunits, *PRKACA* (Cα), *PRKACB* (Cβ), and *PRKARCG* (Cγ) form two isoforms of the PKA holoenzyme, type I and type II, named according to their order of elution in DEAE chromatography and consisting of homodimers of either the RIα and RIβ, or the RIIα and RIIβ subunits, respectively.[8 9] Type I PKA is the physiological mediator of cAMP actions; in basal states, the catalytic subunits bind preferentially to type II regulatory subunits, whereas binding to type I subunits is favoured in stimulated states.[8–11] Studies, mostly in cancer cell lines, have shown RIα overexpression;[8 11] however, animal models with increased RIα levels, such as the *Prkar1b*$^{-/-}$, *Prkar2a*$^{-/-}$, and *Prkar2b*$^{-/-}$ mice, have not shown an increased frequency of tumours.[9 12 13] Although young heterozygous mice of a mixed genetic background did not have an abnormal phenotype,[9 14] we recently showed that *Prkar1a*$^{+/-}$ mice develop mesenchymal tumours at an older age.[15] We also reported development of a variety of tumours in a transgenic (Tg) mouse carrying an antisense sequence for exon 2 of the *Prkar1a* gene (X2AS-RIα) under the control of a tetracycline responsive promoter regulated by the tetracycline transactivator (tTA) (the *Tg(Prkar1a*x2as)1Stra*, *Tg(tTAhCMV)3Uh* mouse line, or the tTA/X2AS mice, which we used as a model of *Prkar1a* downregulation).[16]

In the present investigation, we extend the above observations by presenting a complete phenotyping and biochemical characterisation of the tTA/X2AS mouse and comparing it with histopathology and similar analysis of tissues from patient with Carney complex and *PRKAR1A* inactivating mutations.[17–23]

METHODS

Protocols for human and animal research on Carney complex

The institutional review board (IRB) and animal care and use committee (ACUC) of the National Institute for Child Health and Human Development (NICHD) have approved protocols 95CH0059 and ASP 01–003 for human and animal studies,

Abbreviations: ACTH, adrenocorticotrophic hormone; BAC, bacterial artificial chromosome; bw, bodyweight; CNC, Carney complex; FISH, fluorescent in situ hybridisation; NIH, National Institutes of Health (USA); PKA, protein kinase A; PPNAD, primary pigmented nodular adrenocortical disease

respectively, on CNC. All human specimens studied in the present investigation were obtained under 95CH0059 after obtaining informed consent from CNC patients. The clinical profile of the patients from families CAR01 (YC01) and CAR110 have been reported previously.[1 2] The mouse construct has been described elsewhere.[16]

Mouse dissection and phenotype analysis

For all analyses, the tTA mice and the corresponding MEF lines were considered the control group. All mice were fed and maintained similarly in an inhouse animal facility; they were weighed weekly. For phenotyping, age and sex matched tTA and tTA/X2AS mice were killed approximately every 2 months, from 4 to 18 months of age. Tissue fragments were snap frozen and stored at −70℃ until processed for PKA assay activity, and mRNA and protein studies. Most of the tissue from each organ was fixed and paraffin embedded for routine histological analysis using haematoxylin and eosin staining and PKA subunit immunostaining, following protocols described elsewhere.[4 5] Pathological lesions were further characterised by staining with appropriate tissue markers. Most histopathological analysis was performed in our laboratory and at Molecular Histology, Inc., (Gaithersburg, MD, USA) or at animal facilities at the NIH (Frederick Cancer Research and Development Center, National Cancer Institute, Frederick, MD, USA and the Division of Veterinary Resources, NIH, Bethesda, MD, USA). Tumour cells were prepared from freshly obtained or frozen specimens (touch preparations) and fixed on siliconised slides for genetic analysis (see below).

Dexamethasone administration and corticosterone assays

From the two groups (tTA/X2AS, n = 10 and control tTA, n = 10] 6 month old mice underwent a 6 day long, low and high dose dexamethasone suppression test based on Liddle's protocol in humans[19] with daily collection at 11.00 of serum for determination of corticosterone. Basal levels of the hormone were collected for 2 days, then dexamethasone (Sigma-Aldrich Inc., St. Louis, MO, USA) was administered by daily intraperitoneal injection: 2 days at 0.25 mg/100 g of body weight (bw), followed by 2 days at 2.5 mg/100 g bw. Corticosterone was measured in duplicate by an enzyme immunoassay, following the manufacturer's instructions (American Laboratory Products Company Diagnostics, Windham, NH, USA). The inter-assay and intra-assay coefficients of variation were <5.4%, and cross reactivities with other steroid hormones were <0.05%.

Electron microscopy of adrenal glands

Adrenal glands were removed, dissected, and fixed for 3 hours in 2% formaldehyde and 2% glutaraldehyde in 0.1 mol/l phosphate buffer, pH 7.3. Tissue slices were fixed for 90 minutes (2% OsO_4 in 0.1 mol/l cacodylate buffer, pH 7.3), dehydrated in ethanol, and embedded in epoxy resin. Ultra-thin sections were stained with uranyl acetate and lead citrate, and examined at 80 kV under a Philips (Eindhoven, the Netherlands) CM 10 electron microscope. Adrenal glands from three tTA/X2AS and three tTA mice were analysed by investigators blinded to the genotypes.

PKA immunostaining, and comparison with human adrenal and thyroid tumours

Human adrenal and thyroid tumours from c.578delTG and c.769C→T *PRKAR1A* inactivating mutation carriers was used for haematoxylin and eosin staining and PKA subunit immunostaining. The mutations of these patients have been published previously: the c.578delTG and c.769C→T mutations are those of kindreds CAR01[1] and CAR110.[2] Touch preparations from these tumours (which had been snap frozen and stored at at −70℃ at the time of their excision) were also used for allelic loss studies (see below). All immunostaining with PKA subunits was performed as previously described[4 5] and graded by at least two of the authors (CAS, JAC).

Analysis of mouse and human tumour preparations for allelic losses

For the detection of *Prkar1a* and *PRKAR1A* allelic losses, fluorescent in situ hybridisation (FISH) was performed.[1 4 5] The probes used in both cases were bacterial artificial chromosome (BACs) 172A4 and 321-G8, containing the mouse *Prkar1a* and human *PRKAR1A* genes, which were obtained from commercially available mouse and human BAC libraries, respectively. We have previously described the use of BAC 321G8 for the detection of 17q22-24 allelic losses in human tumours.[4 5] Both BACs were labelled by nick translation with digoxigenin-11 dUTP (Roche Molecular Biochemicals, Indianapolis, IN, USA) for 2.5 hours at 15℃. Fluorescence images were automatically captured and merged using IPLab Spectrum software (Scananalytics Inc., Fairfax, VA, USA) on a PowerPC 8500/150. Overall, at least 100 interphases with strong hybridisation signals per sample were scored. Presence of more than 25% cells with only one *BAC* signal was interpreted as an allelic deletion. Control experiments for CNC tumours, including hybridisation of BAC clones from chromosomes 1, 4, 16, and 22, showed the two expected signals in more than 90% of the cells. Control experiments with tissues from tTA mice showed fewer than 6–12% of cells with only one *Prkar1a* signal; thus, any sample with <12% allelic losses were considered normal. A BAC clone from mouse chromosome 3 was also used as control for FISH with some mouse tumours; consistent with the above observation in normal mouse tissues, 8–11% of cells showed one signal depending on the tissue type, with insignificant variation between samples. A chromosome 17 specific centromeric α-satellite probe labelled with biotin-16 dUTP (Vysis Inc., Donners Grove, IL, USA) was used for chromosome identification. Fluorescence images were automatically captured and merged using IPLab Spectrum software (Scananalytics Inc.) on a PowerPC 8500/150, as described above.[4 5]

PKA activity determinations

PKA assays on mouse tissues were performed as previously described.[1 4 5] For PKA subtyping, DEAE chromatography was used.[24 25] All determinations of PKA activity were performed in duplicate, corrected for protein content, and an average value was calculated for each experiment. Human adrenal tissue homogenates from 3 c.578delTG *PRKAR1A* mutation carriers were also used for DEAE analysis, as described above and elsewhere.[1 4 5]

Statistical analyses

For all analyses, mice expressing tTA only were the control group. Weight comparisons over time were performed by one way analysis of variance followed by Tukey's multiple comparisons test; $p<0.05$ was considered significant. PKA assay data from all tissues were compared with Statistica software (StatSoft, Inc., Cary, NC, USA) using Student's *t* test for individual comparisons between the two mouse lines. All data are shown as mean (SEM). For PKA subtyping, the activity of individual chromatographic fractions was compared by *t* test. In all experiments, $p<0.05$ was considered significant; $p<0.1$ was interpreted as showing a tendency towards a significant difference.

RESULTS

RIα protein levels in tTA/X2AS mice decreased by 60% and 40% in liver and adrenal tissue, respectively (fig 1A); Western blot analysis from kidney and adrenal tissues were also consistent with an approximately 50% decrease of RIα protein levels in these mice compared with the same tissues from matched tTA contols (fig 1). The RIIβ protein was increased in tissue lysates from the tTA/X2AS mice, whereas RIβ, RIIα, and Cα levels were all significantly decreased (fig 1A). These changes in the protein levels of PKA subunits in tTA/X2AS mice were comparable with those in adrenal tumours from patients with CNC and *PRKAR1A* inactivating mutations; these lesions also exhibited an increase in RIIβ (fig 1B). Tissues from the tTA/X2AS mice showed significant PKA activity changes. Overall, there was a marked increase in type II PKA activity (fig 2) with a corresponding increase in the ratio of type II to type I activity (fig 1c). This observation was similar to the type I to type II PKA activity switch that was observed in human adrenal tissue from CNC patients and c.578delTG *PRKAR1A* inactivating mutation carriers (fig 1D).

At birth, tTA/X2AS mice were not physically different from control animals; they gained weight and length normally. Although as a group, tTA/X2AS mice weighed less than their age and sex matched controls during their first weeks of life ($p<0.05$) (fig 3A), there were no weight differences between the two groups during the ensuing 12 months. Control mice reached a plateau in their weight gain by approximately 6 months of life, while tTA/X2AS mice continued to gain weight, thus this group had become significantly ($p<0.05$) heavier by 14 months of age (fig 3B). This weight gain was largely due to the accumulation of vast amounts of lower abdominal visceral fat that often resulted in inguinal hernias (fig 3C). Because visceral fat accumulation is a feature of Cushing's syndrome in CNC patients with *PRKAR1A* inactivating mutations and PPNAD, corticosterone levels were measured in tTA/X2AS mice before and after the administration of low and high doses of dexamethasone; tTA/X2AS mice had higher levels of corticosterone before, during (data not shown), and after the administration of dexamethasone (fig 3D).

Histological abnormalities in the thyroid and adrenal glands, lymphoproliferative disease, and mesenchymal tumours developed in some mice as early as 4–6 months of age.[16] Death rate differences became significant after 16 months of age;[16] the most frequent cause of death was a pulmonary or kidney condition related to a lymphoma, histiocytic sarcoma, another lymphoproliferative or histioproliferative syndrome, or a mesenchymal tumour.

Thyroid follicular hyperplasia, adenomas, and cysts were present in 40% of the tTA/X2AS mice but in none of the controls. Mouse thyroid adenoma cells demonstrated loss of the chromosome *Prkar1a* 11 locus and decreased RIα immunostaining (fig 4A–K). These histological and genetic changes are similar to those seen in the thyroids of CNC patients with the c578delTG and 769C→T *PRKAR1A* inactivating mutations (fig 4L–P).

Adrenocortical abnormalities in the transgenic animals were of two types: although pigment alone was not more frequent in tTA/X2AS mice, cortical hyperplasia with pigment deposits and congestion were seen more frequently in tTA/X2AS mice ($p<0.05$). X zone persistence and hyperplasia, after puberty in males, or multiple pregnancies in females, occurred exclusively in the tTA/X2AS animals and was more prominent in female mice (fig 5A, B). These changes are reminiscent of, but not identical to those of human adrenal glands affected by PPNAD (fig 5C, D). Mouse and human adrenal cells from tTA/X2AS mice and c.578delTG *PRKAR1A* inactivating mutation carriers demonstrated allelic losses of the mouse chromosome 11 *Prkar1a* and human chromosome 17q22-24 *PRKAR1A* loci, respectively (fig 5E–I).

Sections of the adrenal cortex from tTA/X2AS mice with abnormalities such as hyperplasia, congestion, and X zone vacuolisation (fig 5J, K) were examined by EM. Compared with those from tTA mice, tTA/X2AS adrenocortical cells showed cytoplasm with ample smooth endoplasmic reticulum, an increased number of round mitochondria with characteristic tubulovesicular internal membranes that often

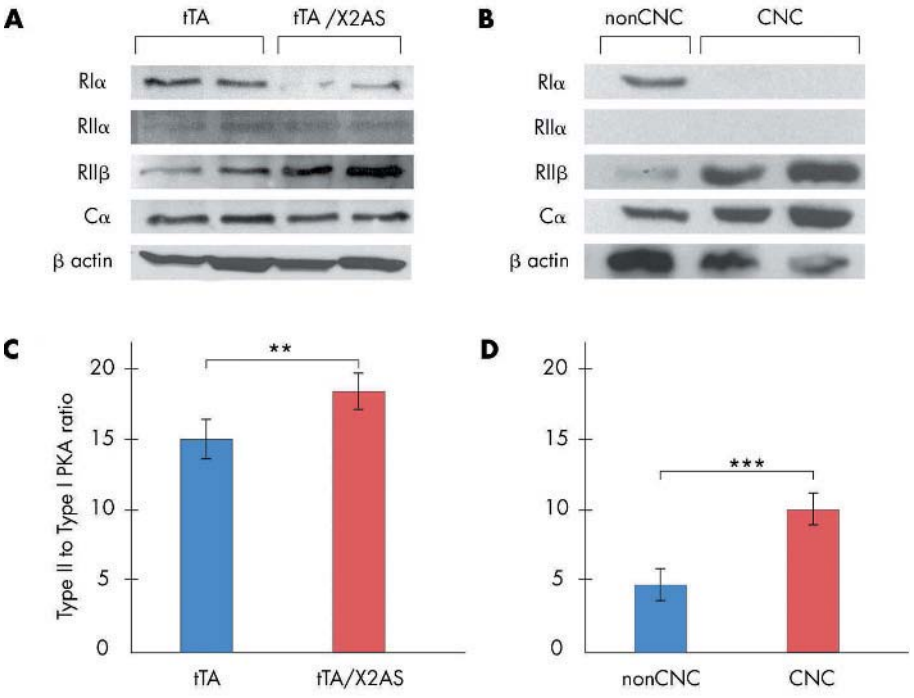

Figure 1 PKA subunit and activity levels after *Prkar1a* downregulation. Decrease in RIα and increase in RIIβ protein levels in mouse (A) and human (B) adrenals from mice with downregulated *Prkar1a* (tTA/X2AS), and from CNC patients with c.578delTG *PRKAR1A* inactivating mutations, respectively. This was associated with an increase in the ratio of type II to type I PKA activities in mouse (C) and human (D) adrenals from tTA/X2AS mice and CNC patients with c.578delTG *PRKAR1A* inactivating mutations, respectively. **$p<0.05$; ***$p<0.001$.

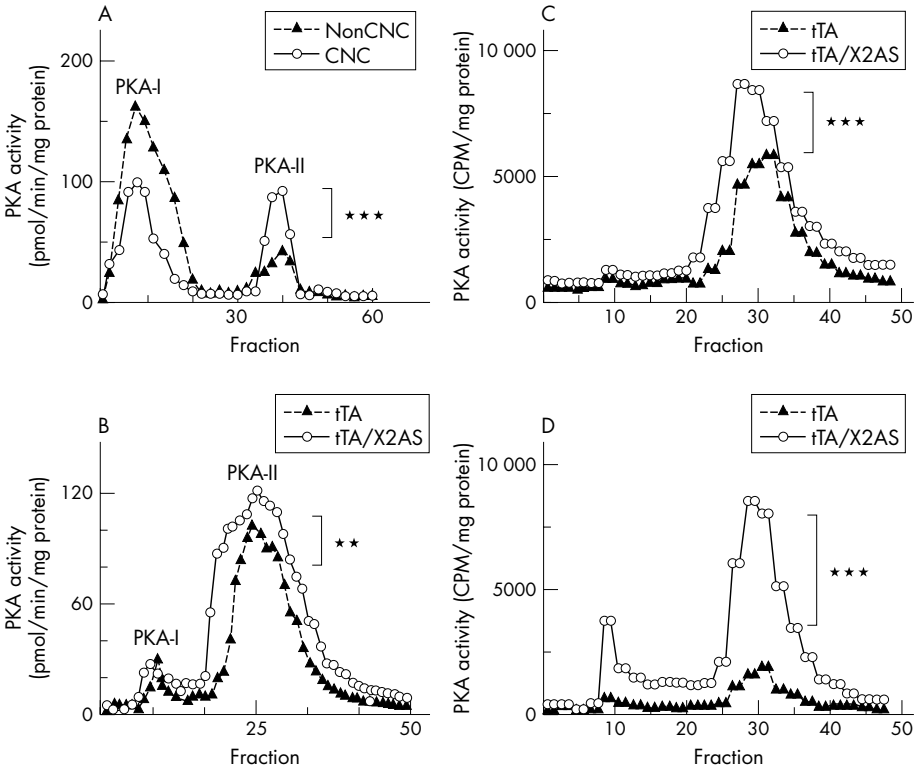

Figure 2 DEAE elution profile of type I and type II PKA activity in human PPNAD from a patient with the 578delTG *PRKAR1A* inactivating mutation (A), mouse adrenal (B), kidney (C), and liver (D) of control and tTA/X2AS mice. A statistical analysis of the type II to type I PKA ratio for the data presented in (A) and (B) is included in fig 4 (C and D) of the main manuscript.A statistical comparison of the type-II PKA peaks between human control and PPNAD, and mouse control and tTA/X2AS adrenals is shown here. **p<0.05; ***p<0.001.

bridged the mitochondrial matrix, and a reduced number of cholesterol storing liposomes (fig 5L, M, and fig 6). Cells from the tTA/X2AS mice also had frequent and large pigment deposits and often other, vesicle-like structures (fig 6B). In addition, in tTA/X2AS adrenal glands, extra-epithelial pigment cells containing melanosomes were found in direct cellular contact with adrenocortical cells (fig 5M, arrows).

Immunostaining of tTA/X2AS adrenocortical cells from areas with the above abnormalities showed a decrease in RIα (fig 7A, B, P, R) and an increase in RIIβ (fig 7K, L, Q, S), but

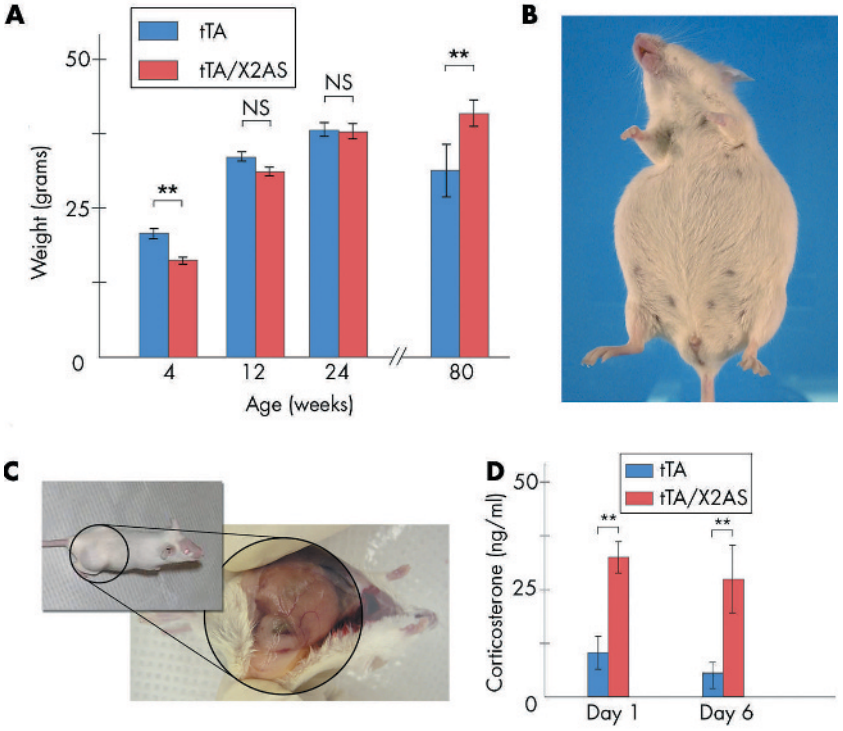

Figure 3 Obesity, hernias and increased corticosterone levels in mice with the downregulated *Prkar1a* gene product (the tTA/X2AS mice). (A) As a group, tTA/X2AS mice weighed less than their age and sex matched controls during their first weeks of life; later, there were no weight differences between the two groups until as control tTA mice reached a plateau in their weight gain by approximately 6 months of life, tTA/X2AS mice continued to gain weight and finally became significantly heavier (B–C) Abdominal obesity and visceral fat accumulation led to frequent inguinal herniation in these mice. (D) tTA/X2AS mice had significantly higher levels of corticosterone both before and after the administration of low and high doses of dexamethasone. **p<0.05.

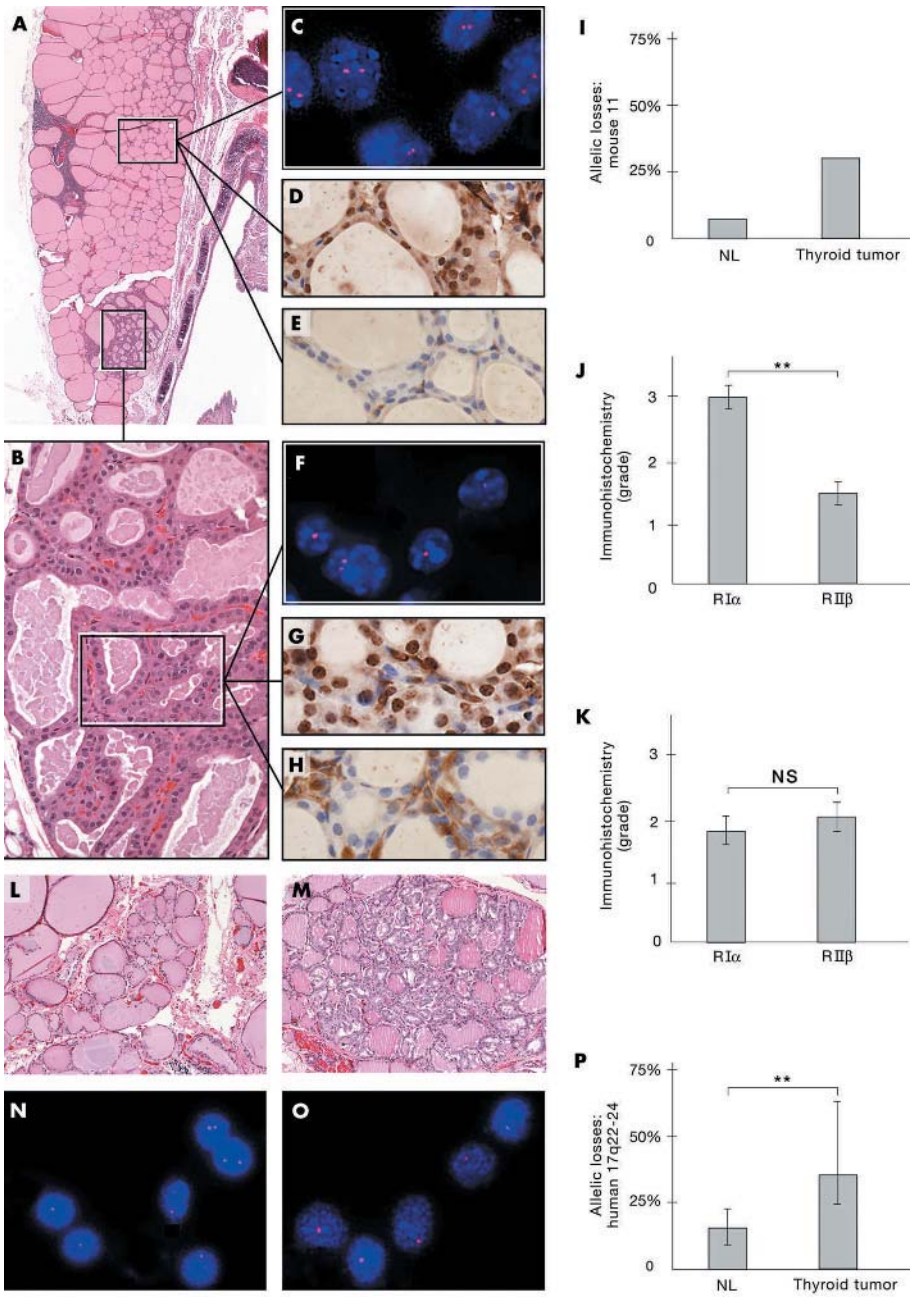

Figure 4 Thyroid lesions in mice with downregulated *Prkar1a* levels and in humans with inactivating *PRKAR1A* mutations. (A) The right lobe of the thyroid gland from a tTA/X2AS mouse shows both normal and abnormal tissue (haematoxylin and eosin staining, ×20). (B) The abnormal tissue shows follicular hyperplasia or early adenoma (haematoxylin and eosin staining, ×40). (C) Almost all cells from normal thyroid tissue show two copies of the mouse chromosome 11 *Prkar1a* locus by in situ hybridisation. (D) Follicular cells from normal mouse thyroid tissue show relatively homogeneous staining for RIα and (E) no staining for RIIβ. (F) Cells from areas of adenomatous tissue show mostly one copy of the mouse chromosome 11 *Prkar1a* locus, and (G) a concomitant decrease in RIα staining and (H) a relative increase in RIIβ staining. Allelic losses are quantified in (I) for mouse lesions versus normal tissue, although insufficient tissue was available to perform a meaningful statistical comparison. Average grading of immunohistochemical staining is shown for thyroids from (J) control and (K) tTA/X2AS mice; overall, there was a decrease in the intensity of the RIα staining but there were no significant changes for that for RIIβ. Only in particular locations (where lesions were forming) and for individual cells, such as the ones shown in (A) (and D, E, G, and H) could the relative increase of RIIβ be appreciated. (L) Thyroid follicular adenoma and (M) papillary in situ carcinoma in patients with CNC and the c.578delTG and c.769C→T *PRKAR1A* inactivating mutations, respectively (haematoxylin and eosin staining, ×20). (N, O) In both these lesions, there is loss of one allele from the chromosome 17 (17q22-24) *PRKAR1A* locus, as quantified in (P) by assessing multiple touch preparations from normal and tumorous tissues.

no changes in RIIα expression (fig 7F, G); these alterations corresponded to the PKA subunit staining of adrenal glands with PPNAD from c.578delTG carrying CNC patients (fig 7E, J, O, X, Y); this pattern was not observed in either normal human adrenocortical tissue (fig 7C, H, M, T, U) or ACTH induced hyperplastic adrenal cortex (Fig 7D, I, N, V, W).

Histiocytic hyperplasia, sarcomas, and lymphomas also developed in tTA/X2AS mice.[16] Although lymphomas or leukaemias have not been recorded in the US National Institutes of Health (NIH) and Mayo Clinic registry of more than 400 patients with CNC, we were prompted by the tTA/X2AS mouse data to investigate this, and identified two cases

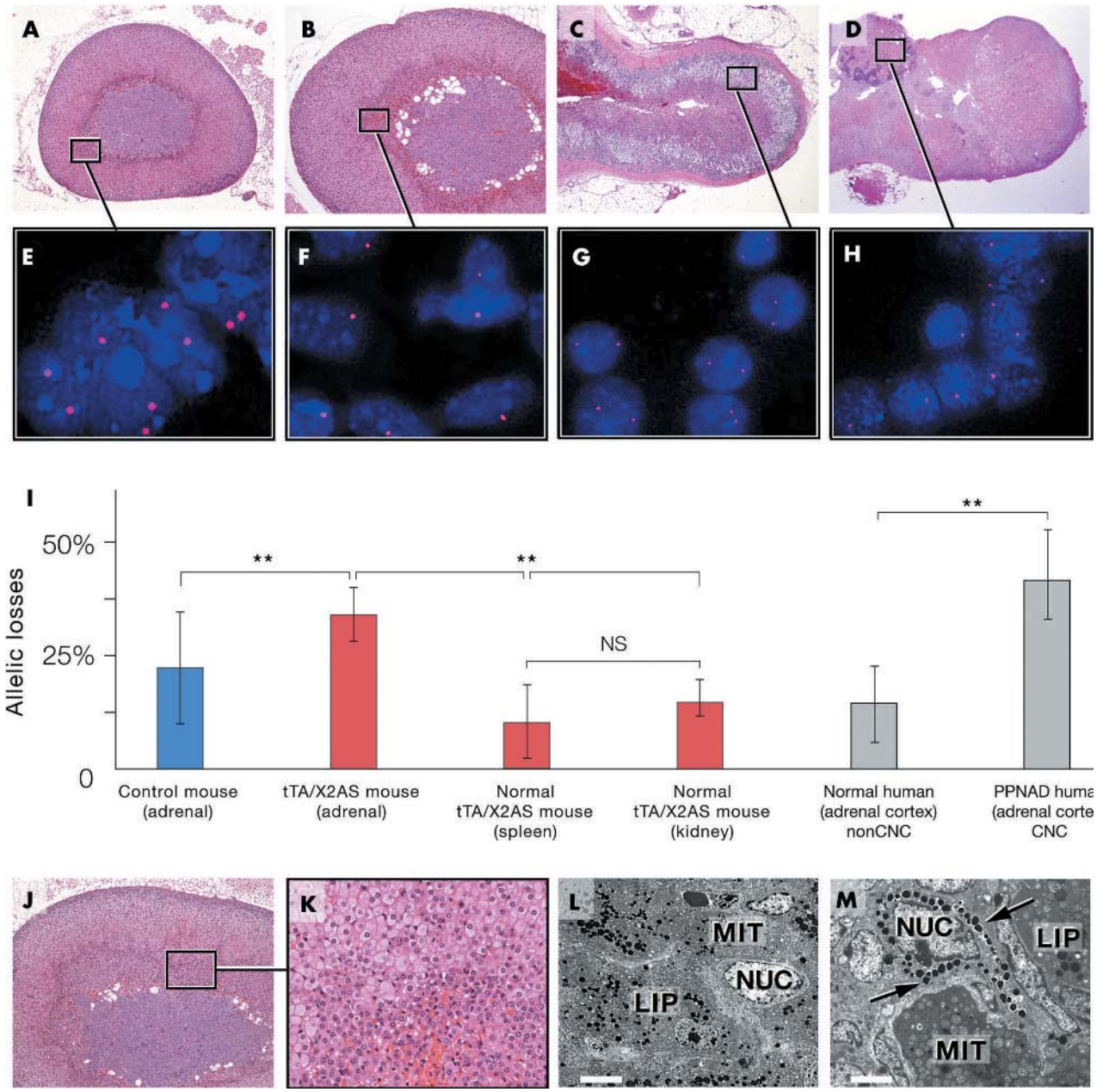

Figure 5 Adrenal lesions in mice with downregulated *Prkar1a* levels and in humans with inactivating *PRKAR1A* mutations. (A) Control female mouse adrenal gland shows reduction of the X zone (located between cortex and medulla); (B) An adrenal gland from an age matched, multiparous female tTA/CNC mouse is larger and shows hyperplasia of the X zone (haematoxylin and eosin staining, ×10). (C) Normal human, and (D) an adrenal gland from a patient with PPNAD and the c.578delTG *PRKAR1A* inactivating mutation showing the characteristic nodular hyperplasia (×5). (E) Cells from the adrenals of control mice show mainly two copies of the mouse chromosome 11 *Prkar1a* locus. (F) Cells from the adrenals of tTA/X2AS mice show mainly one copy of the mouse chromosome 11 *Prkar1a* locus. (G) Cells from the cortex of normal adrenals show two copies of the chromosome 17 (17q22-24) *PRKAR1A* locus. (H) Cells from the nodular cortex of the adrenal gland with PPNAD show loss of one allele from the chromosome 17 (17q22-24) *PRKAR1A* locus. (I) Quantification of allelic losses in mouse and human tissues from tTA/X2AS mice and PPNAD by assessing multiple touch preparations from normal and tissues with lesions; preparations from normal tissues show some allelic losses in the order of 10–20%, which are presumably due to tissue fixation artefacts. Adrenocortical hyperplasia (J; ×10) in the tTA/X2AS mice was also associated with congestion, haemorrhage, pigment deposition, and other histological abnormalities (K; ×40). (L) Electron micrographs of adrenocortical cells in control mice show cytoplasm filled with characteristic round mitochondria with tubulovesicular internal membranes (MIT), ample smooth endoplasmic reticulum, and liposomes (LIP). (M) Electron micrographs of adrenocortical cells in tTA/X2AS mice show cytoplasm with an increased number of large mitochondria (MIT) consistent with their increased corticosterone production, a reduced number of cholesterol storing liposomes (LIP), pigment and marked melanosis (arrows). NUC, nucleus. Bar = 5 μm.

of ectopic, hyperplastic thymic tissue among the four patients with CNC and thyroid tumours who underwent surgery at the NIH (fig 8). In addition, large, macroscopically visible, and occasionally metastatic tumours grew in tTA/X2AS mice.[16] Most of these lesions were of mesencymal origin and corresponded to tumours seen in CNC patients (fig 9).

DISCUSSION

Our study extends our previous findings,[16] and those of Amieux *et al*[14] and Kirschner *et al*.[1 15] Overall, there were significant similarities between the phenotype of tTA/X2AS mice and CNC patients. Firstly, there was a high incidence of thyroid lesions in the tTA/X2AS mice, which are extraordinarily rare in wild type

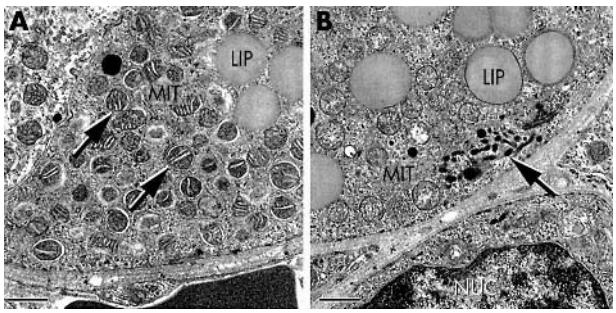

Figure 6 Electron micrographs of adrenocortical cells from tTA/X2AS mice. (A) Significantly more mitochondria than liposomes (LIP) are present in tTA/X2AS adrenocortical cells. The internal membranes of mitochondria (MIT) frequently bridge the mitochondrial matrix (arrows). (B) In close proximity to the plasma cell membrane, a subset of adrenocortical cells in tTA/X2AS demonstrate pigment deposition and vesicle-like structures (arrow). Bar = 1 μm.

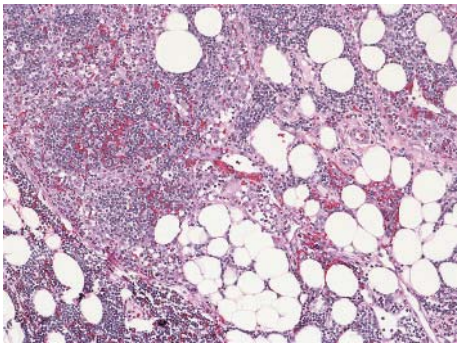

Figure 8 Haematoxylin and eosin staining of ectopic thymic tissue (×40) found in the thyroid of a patient with CNC, a carrier of the c.769C→T *PRKAR1A* inactivating mutation; his thyroid tumour is shown in fig 7L.

animals but frequent among CNC patients.[17 18] Similar to thyroid tumours with *PRKAR1A* mutations and loss of the corresponding chromosome 17q22-24 *PRKAR1A* region, thyroid lesions from tTA/X2AS mice demonstrated loss of the *Prkar1a* locus on mouse chromosome 11 (fig 4).

Secondly, the tTA/X2AS mice had mild hypercorticosteronaemia that led to a chronic Cushing's syndrome-like

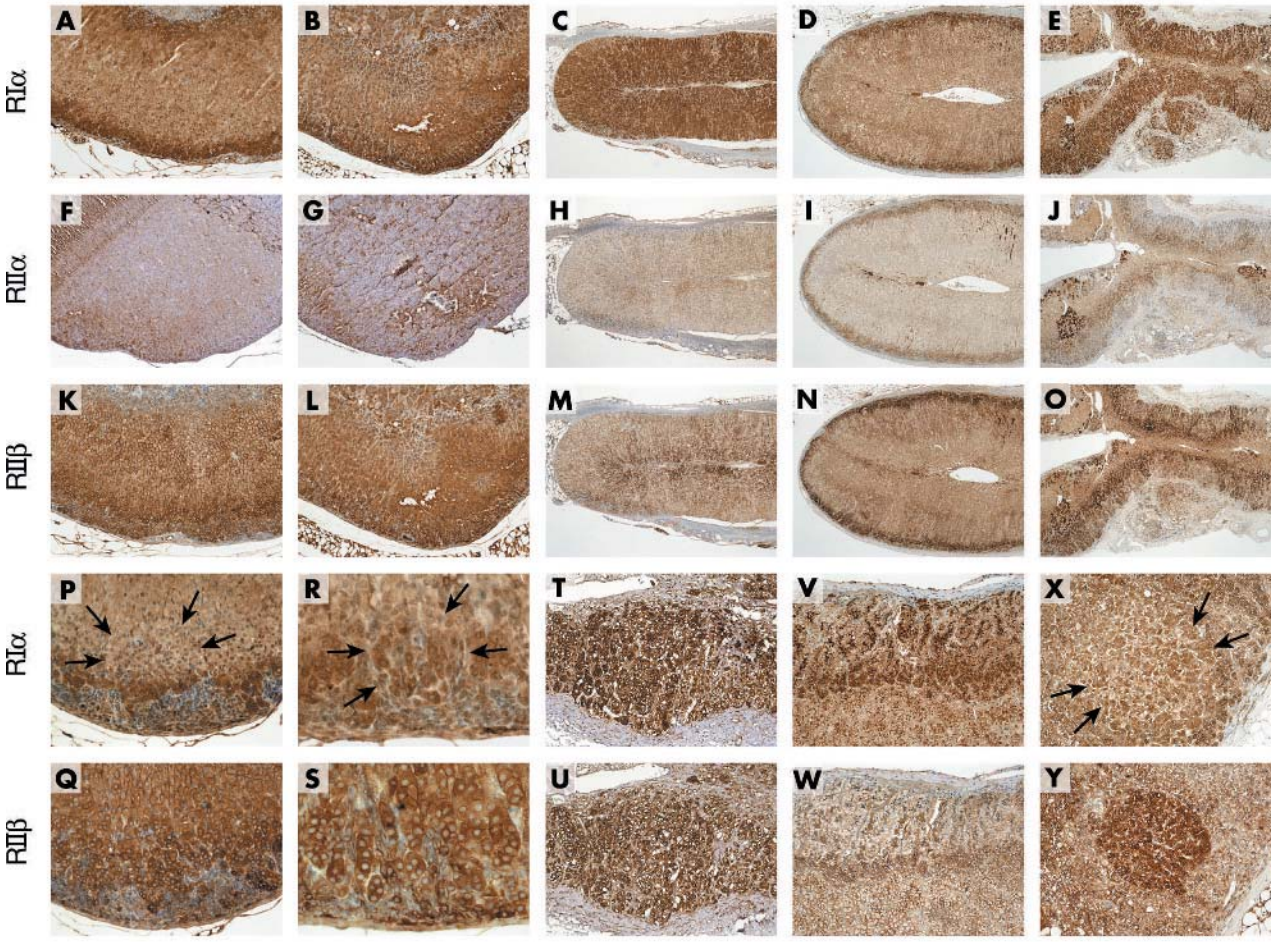

Figure 7 PKA subunit immunostaining of adrenal cortex from control mice (A, F, K; ×20), tTA/X2AS mice (B, G, L; ×20), normal human adrenal cortex (C, H, M; ×10), ACTH induced hyperplasia (D, I, N; ×10), and primary pigmented nodular adrenocortical disease (PPNAD) from a patient with the c578delTG *PRKAR1A* mutation (E, J, O; ×10). (A–E) RIα, (F–J) RIIα, and (K–O) RIIβ. RIIβ stains more intensely in hyperplastic or nodular tissue. Higher magnifications are shown, stained for RIα (P, R, T, V, X) and RIIβ (Q, S, U, W, Y) from tTA/X2AS mice (P, Q, R, S ×40), human normal adrenal cortex (T, U, x20); cortex from ACTH induced hyperplasia (V, W; ×20), and PPNAD (X, Y; ×20). The arrows point to nodules forming in tTA/X2AS mouse adrenocortical tissue (P, R), which become apparent with the relative decrease in RIα staining, whereas RIIβ staining is either normal or increased. In normal or hyperplastic human adrenal cortex, there were no changes in RIα or RIIβ staining; in contrast, in PPNAD, arrows point to an area of decreased RIα staining (X) that corresponds to an adrenocortical nodule clearly identified by the increased RIIβ staining (Y).

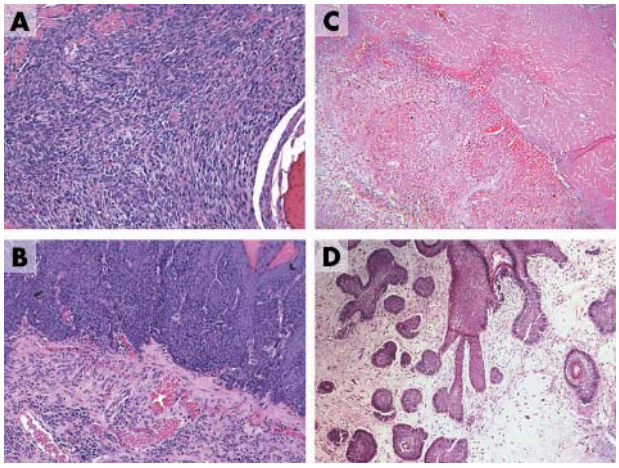

Figure 9 Haematoxylin and eosin stainings of mesenchymal tumours from the tTA/X2AS mice: (A) spindle cell, schwannomatous tumour that had metastasised to the lungs (×20), and (B) skin squamous cell papilloma (×20); these tumours are analogous (but not identical) to the tumours shown in (C) a metastatis of the lung schwannoma (×20), and (d) skin myxoma (×20), from two patients with CNC.

phenotype associated with the persistence and hyperplasia of the X zone of the adrenal cortex. In CNC patients with PPNAD since childhood, mild, atypical, or periodic symptoms due to subtle hypercortisolaemia are more frequent than frank Cushing's syndrome.[19] PPNAD has been postulated to originate from the cells between the mature zonae and medulla; the mouse X zone, in such a location, is the equivalent of the human fetal zone.[20] Interestingly, there were significant EM similarities between tTA/X2AS adrenal cortex and PPNAD: in the latter, an array of small pigmented nodules are often seen, which stud the cortex and range in colour from light grey to grey brown, dark brown, or jet black. The intracytoplasmic pigment has the ultrastructural morphology of lipofuscin, but because treatment of sections with $KMnO_4$ abolishes the pigmentation, it may have a melanin component as well.[21] Although adrenals in the mutated mice did not show the characteristic pigmented nodules of PPNAD macroscopically, there was a conspicuous increase in cortical pigmented cells (figs 5 and 6). These cells had features of both melanosome and lipofuscin-type organelles. In addition, a subset of adrenocortical cells presented secretory granule-like structures that have been described previously in human synaptophysin positive adrenocortical tumours, including PPNAD. As in humans with PPNAD, adrenocortical lesions in these mice most probably started with polyclonal proliferation of individual cells due to deficient *Prkar1a* action. We have shown in human PPNAD tissue that this is followed by the accumulation of additional genetic ''hits'',[22] one of which is the loss of the normal *Prkar1a* allele, as shown by the allelic loss studies (fig 5I). We speculate that this may well occur nearly simultaneously in multiple clones, as appears to be the case in PPNAD associated nodules.[22]

Thirdly, the tTA/X2AS mice developed mesenchymal and epithelial hyperplasias in a variety of tissues, including histiocytosis in multiple organs to glandular ectasia, spindle cell schwannoma, and squamous papilloma tumours (fig 7). CNC patients also develop mesenchymal tumours, as typified by myxomas;[23] these are the most frequent non-endocrine tumours in CNC. Although the tTA/X2AS mice did not develop myxomas, some of their lesions were analogous to some of the rarer tumours that patients with CNC develop, such as ductal adenomas of the breast and trichofolliculoepitheliomas.

Fourthly, the pattern of alterations of PKA activity in liver, testes, and adrenals from tTA/X2AS mice were generally similar to those seen in tumours from CNC patients, or with spontaneous mutations in the *PRKAR1A* gene (figs 1 and 2).

The greatest difference between the phenotypes of CNC patients and tTA/X2AS mice was that of the frequent lymphoid hyperplasia and lymphomas in the latter. However, laboratory mice of many backgrounds (including those of the C57BL/6 background that was used here) tend to develop lymphomas and other haematological pathology more frequently than other types of tumours.[26] Indeed, *Tp53* mutant mice develop lymphoproliferative disease prior to findings suggestive of Li-Fraumeni or other human syndromes caused by *TP53* mutations.[27] While CNC patients are not known to develop lymphomas (in a database that we maintain of more than 400 patients with the disease, none has died of a haematological malignancy[15]), a number of patients with Cushing's syndrome due to PPNAD evaluated at the NIH Clinical Center have been noted to have enlarged thymus on chest MRI (unpublished observations). Fig 8 shows the histology of hyperplastic thymic tissue from the thyroid of the patient that had the tumour shown in fig 4L and was a carrier of the c.769C→T *PRKAR1A* mutation. Of the four CNC patients with thyroid tumours that have been operated at the NIH Clinical Center over the last 20 years, two had ectopic thymic tissue such as the one shown in figure 8. This is an otherwise extraordinarily rare event.[28] It should be noted that both patients also had PPNAD and subclinical Cushing's syndrome at the time of their thyroid operation, which should have made their thymic tissue atrophic.[29]

There are also some subtle differences in the amounts of type I versus type II PKA activities between this mouse model and CNC tumours. While there is a minimal decrease in the type I peak in the tTA/X2AS mouse, there is a dramatic increase in the type II activity (fig 2). One of the significant problems of any effort to reproduce the CNC PKA biochemical phenotype in rodents is the apparent greater percentage of type II PKA activity in murine cells.[11 14] This may make the decrease in type I activity more difficult to detect, particularly as free catalytic subunits, which would be expected to increase in any state of *Prkar1a* down-regulation, co-elute with the type I peak.[11 14] Despite these problems, the tTA/X2AS mice showed the expected further increase in type-II PKA activity in almost all tissues (figs 1 and 2).

What is the molecular mechanism leading to tumours in these mice? Their tissues showed an abnormal cAMP response, a switch to mostly type II PKA activity and an increase in RIIβ subunit. It has been suggested that type I PKA is associated with growth and proliferation, whereas type II PKA is associated with increased differentiation and decreased proliferation.[8 11] However, primary cultures of melanocytes and mammary cells (with mostly type II PKA) are stimulated by cAMP, whereas the mouse Cloudman melanoma and human breast carcinoma lines (with mostly type I PKA) are inhibited by cAMP.[9 30] Furthermore, the switch to type I PKA that was recorded in proliferating cancer cell lines was dependent on high, pharmacologically induced levels of cellular cAMP.[30 31] Indeed, most cells respond to high cAMP levels with inhibition of growth, but some, such as lymphocytes and melanoma cells, are actually stimulated by low cAMP levels.[31] Thus, it is not premature to say that the dysregulated cAMP response of PKA activity in *Prkar1a*$^{-/-}$ and tTA/X2AS cells is at least in part responsible for the pathology we observed. Additionally, there may also be some PKA independent effects of *Prkar1a* that contributed to the phenotype.[32]

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