

# Mouse models of altered protein kinase A signaling

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## Abstract

Protein kinase A (PKA) is an evolutionarily conserved protein which has been studied in model organisms from yeast to man. Although the cAMP–PKA signaling system was the first mammalian second messenger system to be characterized, many aspects of this pathway are still not well understood. Owing to findings over the past decade implicating PKA signaling in endocrine (and other) tumorigenesis, there has been renewed interest in understanding the role of this pathway in physiology, particularly as it pertains to the endocrine system. Because of the availability of genetic tools, mouse modeling has become the pre-eminent system for studying the physiological role of specific genes and gene families as a means to understanding their relationship to human diseases. In this review, we will summarize the current data regarding mouse models that have targeted the PKA signaling system. These data have led to a better understanding of both the complexity and the subtlety of PKA signaling, and point the way for future studies, which may help to modulate this pathway for therapeutic effect.

*Endocrine-Related Cancer* (2009) 16 773–793

## Introduction

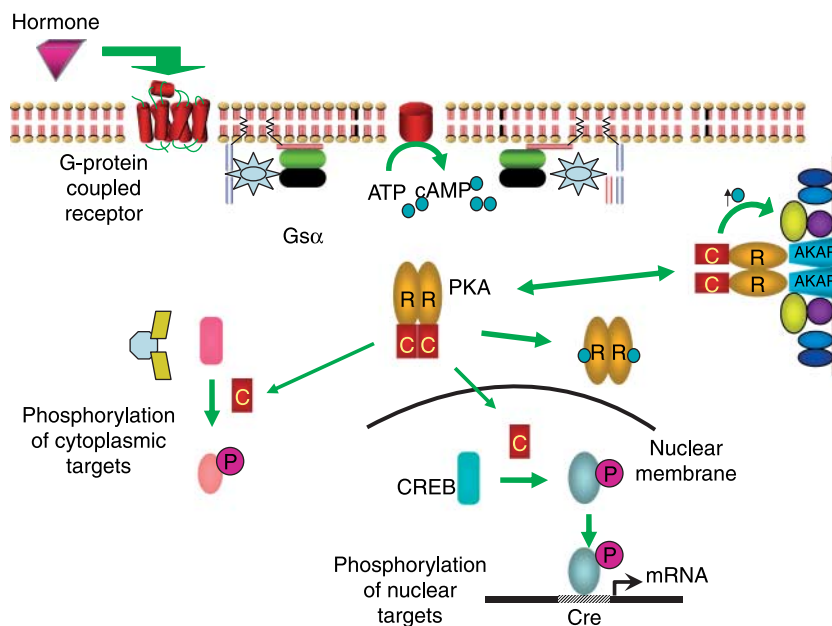
### PKA as a pre-eminent second messenger system

Protein kinase A (PKA) or the cAMP-dependent protein kinase is a serine–threonine kinase that was characterized initially as mediating the effects of elevated cAMP levels, which arose as a result of stimulation of cell membrane by epinephrine (Sutherland & Rall 1958, Walsh *et al.* 1968). These observations formed the basis for understanding hormonal signaling, and provided the basis for two Nobel prize awards in Medicine, to Sutherland (in 1971) and subsequently to Gilman & Rodbell (in 1994). Studies of PKA have formed the basis of signal transduction mediated through protein phosphorylation, and the characterization of this enzyme can be thought to lie at the ‘root’ of recent analysis of all human protein kinases (Manning *et al.* 2002).

In canonical PKA signaling, extracellular hormones bind to G-protein coupled receptors, a large family of seven transmembrane domain proteins involved in many forms of intercellular communication. After liganding of the receptor, a conformational change is

triggered to activate the associated heterotrimeric G-protein. For PKA signaling, these receptors couple to the stimulatory G-protein, Gs. Subsequent dissociation of the Gs complex allows Gs $\alpha$  to associate with membrane bound adenylyl cyclases (ACs), which catalyze the conversion of ATP to cAMP. cAMP binds to PKA, dissociating the inactive holoenzyme and inducing phosphorylation of downstream targets (Fig. 1).

Through early characterization, it was determined that the PKA holoenzyme is comprised of four subunits, two of which are catalytically active (PKAC subunits), and two of each serve to regulate enzyme activity (PKAR subunits). When bound together, the heterotetrameric enzyme is inactive. However, when cAMP levels within the cell rise in response to membrane signaling, cAMP binds cooperatively to paired binding pockets on each of the regulatory subunits, causing release of free, catalytically active, PKAC monomers. Initial studies revealed that manipulation of PKA activity produced changes in the activity of protein substrates (e.g. phosphorylase kinase (Walsh *et al.* 1968)), but also changes in gene transcription, a function mapped to PKA’s ability to



**Figure 1** General overview of protein kinase A signaling. This diagram depicts the canonical PKA signaling pathway. In this model, signaling is initiated by a hormone binding to a transmembrane receptor, activating  $G_{s\alpha}$ , leading to the production of cAMP (blue circles) by adenylyl cyclase. cAMP binds to the PKA heterotetramer, releasing PKAR subunit dimers and free PKAC subunits (red squares). The catalytic subunits phosphorylate both cytoplasmic and nuclear targets (for e.g. CREB), leading to the downstream effects of this intercellular signaling mechanism. At right is shown a PKA–AKAP multiprotein complex which can lead to the phosphorylation of substrates in a highly localized fashion in response to cAMP elevation.

phosphorylate the transcription factor cAMP response element binding protein (CREB; Montminy 1997, Mayr & Montminy 2001, Rosenberg *et al.* 2002), which functionally binds throughout the DNA transcriptome (Conkright *et al.* 2003). A myriad of studies over the past 40 years have identified hundreds (if not thousands) of PKA substrates, both in the nucleus and in the cytoplasm (Budovskaya *et al.* 2005, Huang *et al.* 2007, Neuberger *et al.* 2007, Gao *et al.* 2008). Like other post-translational protein modifications, PKA-mediated phosphorylation can affect any aspect of target protein function, including activity, localization, and stability (Huang *et al.* 2000, Mason *et al.* 2002, Higuchi *et al.* 2003, Qiao *et al.* 2003, Hoang *et al.* 2004, Hino *et al.* 2005, Rolli-Derkinderen *et al.* 2005), such that PKA exerts complex effects on most biologic systems. For this reason, pharmacologic or genetic manipulation in tissue culture cells may produce only a limited understanding of the pleomorphic role of PKA signaling.

To better appreciate the complexity of PKA signaling, significant work has been invested in generating mouse models with genetic manipulation of the PKA system. Although these systems are all to some extent artificial, they allow specific investigation of various aspects of PKA signaling, with a focus on

altered physiology in an intact organism. Thus, these mouse models provide perhaps the best way in which to appreciate how modulation of this system may produce physiological changes, and might profitably be modulated for therapeutic purposes. For the bulk of the review, we will focus on genetic manipulation of the PKA subunits themselves, although other genetic models that affect PKA signaling will also be addressed.

## Components of the PKA signaling system

### Biochemistry of the PKA system

PKA is evolutionarily conserved, such that analysis of PKA function has been carried out in organisms ranging from yeast (Tamaki 2007, Thevelein *et al.* 2008), through *Caenorhabditis elegans* and *Drosophila* (Noveen *et al.* 1996, The *et al.* 1997, Vanfleteren & De Vreese 1997, Angelo & Rubin 1998), to mammals (Skalhegg & Tasken 2000). Because the review of each of these model systems is beyond the scope of this review, we will focus on the human PKA system, and how this relates to the genetically tractable mouse system.

Biochemical studies have identified two distinct isoforms of PKA, which exhibit different physical and biochemical properties. Type 1 PKA is eluted off anion exchange chromatography columns at a relatively low

salt concentration, and exhibits binding affinity for cAMP in the 40–50 nM range (Woodford *et al.* 1989). By contrast, type 2 PKA requires higher salt concentrations to recover the enzyme, and exhibits a slightly lower affinity for cAMP, with affinities in the 50–200 nM range (Robinson-Steiner *et al.* 1984, Rannels *et al.* 1985). Because of both kinetic and affinity parameters of cAMP binding, it is thought that the bulk of the cytosolic PKA activity derives from dissociation of type 1 PKA complexes. However, this does not exclude the likelihood that type 2 PKA plays an important signaling roles owing to very high localized concentrations of cAMP generated at the site of large multiprotein signaling complexes nucleated by A-kinase anchoring proteins (AKAPs; Feliciello *et al.* 2001, Diviani *et al.* 2006).

**PKA catalytic subunits.** At the genetic level, humans have three PKAC subunit genes, designated as *PRKARCA*, *PRKARCB*, and *PRKARCG*. The three genes produce three corresponding  $\alpha$ -,  $\beta$ -, and  $\gamma$  isoforms of the catalytic subunits. Functional studies have not identified significant functional differences among the three isoforms, although there is tissue-specificity of expression. By contrast, there are only two isoforms of PKA catalytic subunits found in mice –  $C\alpha$  and  $C\beta$  (encoded by *Prkaca* and *Prkacb*). Of these two isoforms, there also exist splice variants that have similarities to those found in humans. The  $C\alpha$  subunit consists of two splice variants defined as  $C\alpha 1$  and  $C\alpha 2$ , also referred to as  $C\alpha$ -s (Uhler *et al.* 1986a,b, San Agustin & Witman 2001). Although the  $C\alpha 1$  isoform is expressed ubiquitously in mice, the expression of  $C\alpha 2$  is restricted to male germ cells, and it was shown that  $C\alpha 2$  is the only catalytic subunit of PKA expressed in mature sperm (Desseyn *et al.* 2000).  $C\beta$ , on the other hand, has three splice variants –  $C\beta 1$ ,  $C\beta 2$ , and  $C\beta 3$  (Uhler *et al.* 1986a,b, Guthrie *et al.* 1997).  $C\beta 1$  is ubiquitously expressed, albeit at lower levels than  $C\alpha 1$ , however,  $C\beta 2$  and  $C\beta 3$  expressions are limited to the brain. More specifically,  $C\beta 2$  expression is highest in the limbic regions of the brain, whereas  $C\beta 3$  is more diffusely expressed throughout various brain regions at low levels (Guthrie *et al.* 1997). While the  $C\beta$  isoforms comprise the major PKAC component in the brain,  $C\alpha 1$  is considered to be the predominant source of PKA catalytic activity in non-neuronal tissues (Uhler *et al.* 1986a,b).

Both catalytic isoforms exhibit alternative splicing events at the first exon, resulting in amino-terminal differences between transcript variants. Interestingly, the  $C\alpha 1$  and  $C\beta 1$  isoforms exhibit around 91% homology, and they are both myristoylated at the N-terminus

(Uhler *et al.* 1986a,b, Yonemoto *et al.* 1993, Guthrie *et al.* 1997, Desseyn *et al.* 2000). Conversely,  $C\alpha 2$ ,  $C\beta 2$ , and  $C\beta 3$  are all non-myristoylated due to splicing of alternative first exons that provide novel translation start sites and result in truncated amino termini (Guthrie *et al.* 1997, Desseyn *et al.* 2000). It is thought that N-myristoylation of  $C\alpha 1$  and  $C\beta 1$  is not important for membrane tethering, as is commonly assumed with this type of co-translational modification; rather it is important for protein structural integrity and thermal resistance (Yonemoto *et al.* 1993). However, the lack of myristoylation in the truncated isoforms suggests that this modification is not essential for protein stability.

**PKA regulatory subunits.** For the regulatory subunits, there are four genes, two encoding type 1 subunits and two encoding type 2 subunits. The type 1 subunit genes, *PRKARIA* and *PRKARIB*, exhibit differential expression patterns, with the former expressed ubiquitously, and the latter limited to neural and adipose tissue. The type 2 subunit genes, *PRKAR2A* and *PRKAR2B*, exhibit similar differential expression, although the levels of these two transcripts in most tissues appear generally to be lower than their type 1 counterparts. The  $RII\beta$  subunit is also selectively expressed, being highest in the central nervous system (CNS), neuroendocrine tissues, Sertoli cells, ovarian granulosa cells, and lung (Skalhegg & Tasken 2000). In mice, the PKA regulatory system uses the same genetic complement, with the corresponding mouse genes known as *Prkar1a*, *-r1b*, *-r2a*, and *-r2b*. The expression patterns in mice have been better studied than in human subjects, with similar findings to those described above. For the *PRKARIA/Prkar1a* genes, there is alternative splicing at the 5' end of the gene. This allows the usage of multiple promoters; however, given the fact that the initiation ATG is located in the second exon, the encoded proteins are identical (Solberg *et al.* 1997, Barradeau *et al.* 2000).

**PKA inhibitors.** In addition to PKAC and PKAR subunits, PKA activity is also modulated by the PKA inhibitor (PKI) proteins, which are small, heat stable proteins that are capable of binding and inhibiting PKAC directly. In humans and mice, there are three PKA genes (*PKIA*, *PKIB*, and *PKIG*). The three PKI isoforms appear to exhibit similar biochemical function but different tissue-specific patterns.  $PKI\alpha$  is expressed primarily in muscle, whereas  $PKI\beta$  is expressed at very high levels in the testes. The third gene family member,  $PKI\gamma$ , exhibits ubiquitous expression, but seems to be present at lower levels than the first two.

### A-kinase anchoring proteins

Early biochemical characterization of the PKA signaling system noted that PKA activity could be partitioned into the cytosolic (soluble) and membrane bound (particulate) fractions that exhibited differential enzymatic behavior (Corbin *et al.* 1977). The search for the molecular basis of this observation led to the identification of AKAPs, a family of proteins that bind to PKA and enable its subcellular distribution. This topic has been extensively reviewed (Michel & Scott 2002, Ruehr *et al.* 2004, Dell'Acqua *et al.* 2006, Beene & Scott 2007, Jarnaess & Tasken 2007, Dodge-Kafka *et al.* 2008) and will not be covered in the depth; however, a few points are worth highlighting.

Although there are only 15 AKAP genes in humans, most of the genes exhibit alternative splicing, so that > 50 distinct proteins have been described. In addition to these genes, proteins with other functions in the cell have been shown to serve as AKAPs, although they were initially characterized for other activities (Jarnaess & Tasken 2007). Examples of this latter class of AKAP include cytoskeletal linking proteins of the FERM family, including both Ezrin (Sun *et al.* 2000) and Merlin (NF2; Gronholm *et al.* 2003), as well as the microtubule-associated protein 2 (MAP2; Harada *et al.* 2002). For most of the AKAPs, the binding affinity for type 2 PKA is significantly higher than for type 1, although some AKAPs (particularly AKAP1/D-AKAP1 and AKAP10/D-AKAP2) appear to be able to bind both type 1 and type 2 with reasonable affinity.

PKA-AKAP complexes are multiprotein structures that contain the components to provide exquisite signal feedback control. In addition to binding PKA, most AKAPs also bind phosphodiesterases of the PDE4 family, which tightly regulate cAMP levels as part of a complex feedback loop. Perhaps the best characterized is an AKAP6 (mAkap)-containing complex, which not only contains PKA and PDE4D3, but the cAMP-responsive protein Epac, Rap1, and ERK5, all of which co-exist within overlapping feedback loops (Dodge-Kafka *et al.* 2005). Other AKAP complexes contain PP1- or PP2-type phosphatases, for regulation of protein phosphorylation state (Michel & Scott 2002, Dell'Acqua *et al.* 2006, McConnachie *et al.* 2006). In addition to the signaling components, PKA-AKAP complexes are typically localized to PKA-regulated targets, including membrane channels and receptors. As PKA-mediated phosphorylation of these target proteins affects their function within the cell and thence cell behavior, it is inherently obvious why so much cellular effort goes into the exquisite spatiotemporal regulation of PKA activity at these sites.

### Relation to human disease (and endocrine cancer)

Although PKA signaling has been studied in cells for many years, recent interest in this field has been kindled by the observations that abnormalities of the PKA signaling pathway are associated with human disease. This paradigm was initially discovered in association with the McCune-Albright syndrome (MAS), an endocrine hyperplasia/hyperfunction syndrome caused by activating mutations in the *GNAS1* gene, which stimulates AC and drives PKA activity in cells where the mutant protein is expressed (Weinstein *et al.* 1991, Shenker *et al.* 1994).

Because MAS can be associated with pituitary tumors and hyperfunctioning thyroid nodules (among other manifestations), these tumor types were also evaluated for mutations in the *GNAS1* gene. Mutations in this gene are found in ~33% of GH-secreting pituitary tumors (Vallar *et al.* 1987, Barlier *et al.* 1998), but are uncommon in thyroid tumors, although there have been case reports of these mutations in congenital autonomous goiters (Weinstein *et al.* 2001, Palos-Paz *et al.* 2008). By contrast, activating mutations of the TSH receptor, which signals through *GNAS1*, have been described both in nodules and in congenital cases (Parma *et al.* 1993, 1997, Russo *et al.* 1995, Fuhrer *et al.* 1997a,b, Cetani *et al.* 1999, Postiglione *et al.* 2002, Palos-Paz *et al.* 2008).

Perhaps the most striking demonstration of the association of PKA abnormalities with human disease was the finding within the last decade that the inherited multiple endocrine neoplasia syndrome Carney Complex (CNC) is due to inactivating mutations in the *PRKARIA* gene (Kirschner *et al.* 2000a,b). Although CNC is genetically heterogeneous, mutations of *PRKARIA* appear to account for 60–75% of CNC patients, either in the sporadic or familial setting (Bertherat 2006). Mutation of this gene leads to enhanced PKA activity and tumorigenesis (Kirschner *et al.* 2000a,b). Subsequent to this observation, it has been demonstrated that the same gene can be lost from sporadic adrenocortical tumors (Bertherat *et al.* 2003) or from advanced thyroid cancers (Sandrini *et al.* 2002a,b). These latter tumors exhibit loss of heterozygosity (LOH) at the *PRKARIA* locus in about one-third of the advanced tumors. Although genetic changes in this gene have not been observed in pituitary tumors (Kaltsas *et al.* 2002, Sandrini *et al.* 2002a,b, Yamasaki *et al.* 2003), analysis of protein expression has suggested that it may be down-regulated by epigenetic means specifically in GH-producing tumors (Lania *et al.* 2004).



Most recently, mutations in the *PDE11* and *PDE8* genes, which are involved in the degradation of cAMP, have been detected in adrenocortical neoplasms (Horvath *et al.* 2006, 2008). These findings confirm the strong biological link between PKA activation and hyperproliferation/tumorigenesis.

## Understanding the function of PKA subunits by conventional gene knockout models

### PKA catalytic subunit knockout mouse models

Skalhegg *et al.* (2002) described a mouse in which both the  $C\alpha 1$  and  $C\alpha 2$  subunits were effectively knocked out by replacing exons 6–8 with a neomycin resistance cassette. This targeted deletion of the  $C\alpha$  subunits led to perinatal lethality or delayed growth in mice. Because  $C\alpha 1$  is not only ubiquitously expressed, but also predominantly activated in non-neuronal tissues, the effect on homozygous null (*Prkaca*<sup>−/−</sup>) animals was widespread. Additionally, due to the specificity of  $C\alpha 2$  to the testis, there were also dramatic defects seen in sperm function in these animals.

The most notable defect seen in *Prkaca*<sup>−/−</sup> mice was their delayed growth. Indeed, most mutant animals died perinatally; however, ~27% survived to adulthood. Of this small percentage of mice, there was a marked decrease in overall body size at three months of age, such that mutant mice weighed only about 65% as much as their wild-type (WT) littermates (Skalhegg *et al.* 2002). More recently, it has been shown that the epithelial growth factor receptor protein levels are significantly decreased in *Prkaca*<sup>−/−</sup> mice, which highlight a possible mechanism by which loss of PKA activation potential results in an impaired ability for cells to convey growth signals (Oksvold *et al.* 2008).

In the germ cells of male *Prkaca*<sup>−/−</sup> mice, loss of the catalytic subunits translated to normal testis histology, and normal spermatogenesis as expected, since PKA activity is normally minimal in these cells (Skalhegg *et al.* 2002). However, after maturation, *Prkaca*<sup>−/−</sup> sperm displayed significant malformations in head shape and forward velocity as compared with sperm from normal littermates. These results are interesting, given that the opposite effect is seen in *Prkar1a* heterozygotes, which exhibit fertility problems associated with deficient sperm development and overall reduced sperm count (Burton *et al.* 2006). Taken together, these data represent the fine balancing act of activation and suppression of PKA in order that the appropriate development and function of adult tissues can be achieved.

In contrast to *Prkaca*<sup>−/−</sup> mice, the conventional knockout (KO) mice for  $C\beta 1$  (*Prkacb*[ $\beta 1$ ]<sup>−/−</sup>) are viable, fertile, and phenotypically indistinguishable from their WT littermates (Qi *et al.* 1996). Interestingly, the only differences observed between mice lacking  $C\beta 1$  and normal mice were observed in the hippocampus. Although  $C\beta 1$  null hippocampal slices could be stimulated acutely, they were unable to potentiate electrostimuli along the Schaffer Collateral-CA1 pathway to the same extent as normal hippocampal slices *in vitro* (Qi *et al.* 1996). Additionally, in a process known as long-term depression (LTD), sustained low-frequency stimulation (LFS) results in desensitization of the synapses in normal hippocampal slices, however,  $C\beta 1$  null synapses were not subject to this phenomenon (Qi *et al.* 1996). Given the nature of these defects, and the tissue affected by  $C\beta 1$  mutation, a role for PKA in contextual learning becomes more apparent.

Additional evidence for the role of PKA in learning and memory is found in mice harboring mutations that ablate all three splice variants of  $C\beta$  (*Prkacb*<sup>−/−</sup>). *Prkacb*<sup>−/−</sup> mice carry a targeted deletion of exon 2, which is shared among splice variants, and results in phenotypically normal mice (Howe *et al.* 2002). Although the basal activity for PKA was significantly decreased in the amygdala, hippocampus, and cortex of *Prkacb*<sup>−/−</sup> mice, loss of  $C\beta$  resulted in a compensatory increase in  $C\alpha$  expression, causing the activation potential for PKA in the brain to remain largely unaffected (Howe *et al.* 2002). Activation of PKA in both the amygdala and the hippocampus is known to play a role in learning and memory formation. Despite compensatory up-regulation of  $C\alpha$ , *Prkacb*<sup>−/−</sup> mice were less affected by cued fear conditioning, although these observations appear to be strain specific (Howe *et al.* 2002). Nevertheless, given that *Prkacb*[ $\beta 1$ ]<sup>−/−</sup> mice have impaired hippocampal synapse potentiation and *Prkacb*<sup>−/−</sup> animals display learning defects despite compensation by  $C\alpha$ , it is apparent that the activation of the specific isoforms of PKA catalytic subunits is essential for proper molecular signaling during learning and memory (Table 1).

To further address the role of PKA activation per total in the mouse, complete KO mice for both  $C\alpha$  subunits and  $C\beta 1$  were generated (Huang *et al.* 2002). Complete KO of the catalytic subunits (*Prkaca*<sup>−/−</sup>; *Prkacb*[ $\beta 1$ ]<sup>−/−</sup>) resulted in early embryonic lethality; however, when one allele of either  $C\alpha$  (including both splice variants) or  $C\beta 1$  remained unaffected (*Prkaca*<sup>+/-</sup>; *Prkacb*[ $\beta 1$ ]<sup>−/−</sup> or *Prkaca*<sup>−/−</sup>; *Prkacb*[ $\beta 1$ ]<sup>+/-</sup>), there were significant defects in neural tube development leading to spina bifida and/or

Table 1 Phenotype of protein kinase A (PKA)-C subunit knockout mice

Genotype	Phenotype	Reference
<i>Prkaca</i> <sup>-/-</sup>	Growth deficiencies Male infertility	Skalhegg <i>et al.</i> (2002)
<i>Prkacb[β 1]</i> <sup>-/-</sup>	Phenotypically normal Deficiencies in neuronal signaling in hippocampus	Qi <i>et al.</i> (1996)
<i>Prkacb</i> <sup>-/-</sup>	Phenotypically normal Cued learning deficiencies (strain specific)	Howe <i>et al.</i> (2002)
<i>Prkaca</i> <sup>-/-</sup> ; <i>Prkacb[β 1]</i> <sup>-/-</sup>	Embryonically lethal	Huang <i>et al.</i> (2002)
<i>Prkaca</i> <sup>+/-</sup> ; <i>Prkacb[β 1]</i> <sup>-/-</sup>	Spinal neural tube defects (100%)	Huang <i>et al.</i> (2002)
<i>Prkaca</i> <sup>-/-</sup> ; <i>Prkacb[β 1]</i> <sup>+/-</sup>	Embryonically lethal Spinal neural tube defects (75%) Spinal neural tube defects + exencephaly (25%)	Huang <i>et al.</i> (2002)

exencephaly. Although *Prkaca*<sup>+/-</sup>;*Prkacb[β 1]*<sup>-/-</sup> animals survived birth, all of them exhibited severe spina bifida resulting from failure of vertebral arch fusion at the dorsal midline between the forelimbs and hindlimbs. No *Prkaca*<sup>-/-</sup>;*Prkacb[β 1]*<sup>+/-</sup> animals survived gestation, and all of them exhibited severe neural tube defects leading to spina bifida. A small percentage (25%) of these animals had additional cranial defects that led to exencephaly, although this phenotype was not observed in *Prkaca*<sup>+/-</sup>;*Prkacb[β 1]*<sup>-/-</sup> pups (Huang *et al.* 2002). Intriguingly, both *Prkaca*<sup>-/-</sup>;*Prkacb[β 1]*<sup>+/-</sup> and *Prkaca*<sup>+/-</sup>;*Prkacb[β 1]*<sup>-/-</sup> embryos were histologically indistinguishable when looking at neural tube defects in early development. Also, both genotypes displayed morphological defects in dorsal root ganglia organization, with increased apoptosis and regression by embryonic day 12.5. Altogether, these observations point to an essential role for proper PKA activation that cannot be compensated regardless of the subunit affected. Furthermore, molecular analysis of these models suggests that during neural tube development, the role of PKA in suppressing Sonic hedgehog signaling is paramount for cells to undergo patterning and differentiation into the proper neural precursors that make up the adult spinal column (Huang *et al.* 2002).

It is clear in the cases of PKA catalytic subunit KO mice that precise on/off regulation of the enzyme is imperative for proper development of many of tissues. As mentioned above, in the testis it is essential that PKA is not activated during spermatogenesis, but its activity is vital to the function of mature spermatoocytes. Furthermore, not only is on/off regulation important, but so is the regulation of the proper subunits in specific tissues. The Cβ KO mice are a perfect example of how compensation by the Cα subunit is not enough to rescue the neuronal signaling deficits seen in these animals. Moreover, in the combinatorial KOs where a single allele of either Cα

or Cβ1 is unaffected, the loss of the other three alleles cannot be compensated by the one remaining WT allele, regardless of the isoform. Altogether, these studies are highly insightful, and they elegantly exemplify the true complexities involved in PKA regulation during development and adulthood.

PKA regulatory subunit KO mouse models

Study of the PKA regulatory subunit KO models has reinforced prior knowledge, but has also led to new insights into the function of these gene products. Although described in detail below, this data is summarized in Table 2.

Prkar1a KOs

As noted above, *PRKARIA/Prkar1a* is the most highly and widely expressed regulatory subunit of PKA. When the gene was knocked out from mice by replacing exon 3 of the gene with a Neomycin resistance cassette, homozygous null embryos died early during gestation due to a failure of normal mesodermal development (Amieux *et al.* 2002). Specifically, there was a failure of circulatory structures including the heart tube and other structures of the primitive vasculature. There was also a marked reduction in the size of axial and paraxial mesoderm, implying a generalized defect in mesodermal differentiation. Biochemical investigation of the mutant embryos revealed an enhanced basal PKA activity with a reduction in total PKA, consistent with the previous observation that free PKA has reduced stability within the cell (Hemmings 1986). Intriguingly, introduction of null alleles for *Prkaca* substantially improved the phenotype of *Prkar1a*<sup>-/-</sup> embryos, although it did not restore post-embryonic viability. This observation was interpreted to signify that the majority of the developmental effects were due to excess unregulated PKA activity. These same

developmental defects were observed in mice homozygous for a different null allele for *Prkar1a*, generated by removal of exon 2 from the *Prkar1a* gene (Kirschner *et al.* 2005).

Although *Prkar1a*<sup>-/-</sup> mice were not viable, heterozygotes (*Prkar1a*<sup>+/-</sup> mice) did survive and were predicted to be a good genetic model for studying tumorigenesis associated with the CNC tumor predisposition (Kirschner *et al.* 2000a,b). Although the initial characterization of the animals did not indicate early signs of tumorigenesis (Amieux *et al.* 2002), more lengthy follow-up confirmed the tumorigenic nature of this genetic change. In an analysis of 17 animals up to 19 months of age, Veugelers *et al.* reported tumors in 12 mice (57%) consisting primarily of sarcomas and some hepatocellular carcinomas. In an analysis of a larger group of mice up to 2 years old, our group described a somewhat different pattern of tumors, with a spectrum much more similar to those observed in patients with CNC (Kirschner *et al.* 2005). Specifically, we observed osteochondromyxomas in over 80% of mice, schwannomas in 33%, and thyroid tumors in about 10% of animals. Unlike the pigmented schwannomas observed in CNC, the mouse schwannomas were not pigmented, but exhibited classical features of mouse Schwann cell tumors.

Because both alleles studies were well-defined nulls, it seems unlikely that the differences in the models related to residual *Prkar1a* activity. Rather, it is more likely that the discrepancy is due to strain-specific differences between the models. Also, it appears that some of the tumors characterized by Veugelers *et al.* as calvarial ‘sarcomas with myxomatous differentiation’ may be the same tumors which we have classified as osteochondromyxomas by a combination of histological and immunohistochemical analysis (Pavel *et al.* 2008). Taken together, the complex

phenotype of the *Prkar1a*<sup>+/-</sup> mice indicates that these animals serve as an excellent genetic model for human patients with CNC.

#### Prkar1b KOs

In contrast to the *Prkar1a* KOs, mice lacking *Prkar1b* are viable and fertile, and grossly normal in appearance (Brandon *et al.* 1995). Measurements of PKA activity from the brains of these animals did not reveal significant changes from WT, likely due to compensatory increases in levels of the *Prkar1a* protein (Amieux *et al.* 1997). Because of the well-described association of PKA dysregulation with disruption in synaptic long-term potentiation (LTP; Abel & Nguyen 2008), memory processes were studied in the *Prkar1b*<sup>-/-</sup> mice. This analysis revealed defective hippocampal function, which affected multiple pathways. *Prkar1b*<sup>-/-</sup> animals exhibited deficient LTD at both the Shaffer collateral-CA1 synapse on pyramidal cells and the lateral perforant path-dentate synapse on granule cells. Also, depotentiation at CA1 synapse was also significantly reduced in mutant mice. It was concluded from these studies that *Prkar1b* is specifically required in the brain for LTD and depotentiation, as well as for LFS-induced LTD and may play a role in the long-term effects involving synaptic plasticity (Brandon *et al.* 1995). These findings that complemented the observations from the *Cβ1* KO animals confirmed the role of this pathway in long-term memory affecting the CA3 pathway (Huang & Kandel 1996).

#### Prkar2a KOs

Similar to the observations made with the *Prkar1b* null animals, mice lacking *Prkar2a* were healthy appearing and had no obvious phenotype (Burton *et al.* 1997). As in the *Prkar1a*<sup>-/-</sup> mice, total levels of PKA activity

**Table 2** Phenotype of protein kinase A (PKA)-R subunit knockout mice

Genotype	Phenotype	Reference
<i>Prkar1a</i> <sup>-/-</sup>	Embryonic lethal by e10.5, failure of mesodermally-derived structures, notably heart and vasculature	Amieux <i>et al.</i> (2002) and Kirschner <i>et al.</i> (2005)
<i>Prkar1a</i> <sup>-/-</sup> ; <i>Prkaca</i> <sup>-/-</sup>	Partial rescue of phenotype, with restoration of mesodermal specification and heart formation. Retains embryonic lethality but at a later date	Amieux <i>et al.</i> (2002)
<i>Prkar1a</i> <sup>-/-</sup> ; <i>Prkaca</i> <sup>+/-</sup>	Partial rescue of <i>Prkar1a</i> <sup>-/-</sup> phenotype, but intermediate between <i>Prkar1a</i> <sup>-/-</sup> ; <i>Prkrca</i> <sup>+/-</sup> and <i>Prkar1a</i> <sup>-/-</sup> ; <i>Prkaca</i> <sup>-/-</sup>	Amieux <i>et al.</i> (2002)
<i>Prkar1b</i> <sup>-/-</sup>	Deficient long-term memory pathways	Brandon <i>et al.</i> (1995)
<i>Prkar2a</i> <sup>-/-</sup>	No clear phenotype	Burton <i>et al.</i> (1997)
<i>Prkar2b</i> <sup>-/-</sup>	Lean, resistant to diet-induced obesity. Impaired motor coordination	Cummings <i>et al.</i> (1996) and Brandon <i>et al.</i> (1998)
<i>Prkar2b</i> <sup>-/-</sup> ; <i>Ucp1</i> <sup>-/-</sup>	Lean phenotype retained	Nolan <i>et al.</i> (2004)
<i>Prkar2b</i> <sup>-/-</sup> ; <i>Lep</i> <sup>ob/ob</sup>	Rescue of obese phenotype	Newhall <i>et al.</i> (2005)
<i>Prkar2b</i> <sup>-/-</sup> ; <i>A<sup>y</sup></i>	Rescue of obese phenotype	Czyzyk <i>et al.</i> (2008)

were reduced in the *Prkar2a*<sup>-/-</sup> model, likely associated with post-transcript decreases in PKAC and increases in Prkar1a. Although it was hypothesized that disruption of Prkar2a would disrupt the interaction between PKA and the L-type calcium channels, significant changes in the relative subcellular distributions were not observed, suggesting that other PKAR subunits could substitute for this function of Prkar2a. To specifically probe the role of Prkar2a on CNS function, *Prkar2a*<sup>-/-</sup> mice were analyzed for LTP and LTD in the visual cortex by studying ocular dominance plasticity (Rao et al. 2004). This analysis showed that mutant mice exhibited specific defects in LTP (but not LTD) with a resultant effect on visual cortex plasticity, indicating a unique role for Prkar2a in this process.

### Prkar2b KOs

Mice lacking Prkar2b appeared morphologically normal and were fertile, although it was noted that the mice exhibited decreased adipose tissue (Cummings et al. 1996, Brandon et al. 1998). As in the other PKAR subunit KO models, levels of total PKA activity were reduced in the brains of these animals, resulting from compensation by up-regulation of Prkar1a (Amieux et al. 1997). Because previous analyses had suggested that Prkar2b is the major PKAR subunit in the striatum, efforts were made to characterize the brain function of *Prkar2b*<sup>-/-</sup> mice, particularly in regard to function of the dopaminergic system. *Prkar2b*<sup>-/-</sup> mice showed an impaired ability to coordinate motor output while performing the rotarod task, and they exhibited increased locomotor sensitization to chronic amphetamine administration. Specific molecular changes were also observed in the brains of these animals, including reductions in both c-fos and dynorphin mRNAs. Overall, the CNS defects in *Prkar2b*<sup>-/-</sup> mice are attributable to a combination of relative PKA deficiency and the absence of correct subcellular localization (Brandon et al. 1998). These defects were proposed to affect dopamine-mediated gene expression changes, although acute locomotor effects were preserved.

In the adipose tissue, where Prkar2b is abundant, it was shown that RIIβ-containing holoenzyme has an important role in energy balance and adiposity. The *Prkar2b*<sup>-/-</sup> mice, even if apparently healthy and fertile, have markedly diminished white adipose tissue despite normal food intake. These mice do not develop diet-induced obesity or fatty livers, and consistently exhibit a lean phenotype (Cummings et al. 1996). The explanation for this observation is thought to lie in the

fact that the isozyme switch to type 1 PKA induces a higher rate of metabolic uncoupling, leading to elevations of metabolic rate and body temperature, with enhanced energy utilization. Not only were these mice resistant to changes in body weight, but they also resisted diet-induced development of type 2 diabetes. Study of these animals showed that the RIIβ KO mice have lower body weight, low insulin levels and improved total-body glucose disposal as compared with the WT controls, leading to the conclusion that loss of *Prkar2b* protects mice from diet-induced obesity, insulin resistance, and dyslipidemia (Schreyer et al. 2001).

A more detailed study in adipose tissue looked at the uncoupling protein 1 (UCP1) in *Prkar2b*<sup>-/-</sup> mice. UCP1 is primarily responsible for adaptive thermogenesis in response to sympathetic stimulation of the brown adipose tissue. Mice lacking Prkar2b exhibit nocturnal hyperactivity in addition to the increased oxygen consumption at rest. This study also analyzed *Prkar2b*<sup>-/-</sup>; *Ucp1*<sup>-/-</sup> double KO mice, which retained the lean phenotype. This observation demonstrated that induction of UCP1 and increased resting oxygen consumption are not the cause of leanness in the *Prkar2b*<sup>-/-</sup> mice (Nolan et al. 2004). In addition to this, experiments using leptin-deficient *Lep*<sup>ob/ob</sup> mice showed that the KO of *Prkar2b* provided substantial rescue of the obese phenotype. Basal oxygen consumption and nocturnal locomotor activity were increased, whereas body weight gain and food consumption decreased in the double mutant lacking both leptin and RIIβ (Newhall et al. 2005). A similar finding was made for Agouti lethal yellow (*A<sup>y</sup>*) mice, another model of mouse obesity caused by ectopic expression of the MC4R antagonist Agouti protein (Czyzyk et al. 2008).

### Other generalized KO models affecting PKA signaling

#### PKI KOs

Surprisingly, mice carrying a KO of *Pkia* (*Pkia*<sup>-/-</sup>) showed a fairly modest phenotype, with decreased basal and stimulated gene expression, which was attributed to a compensatory increase in *Pkar1a* levels (Gangolli et al. 2000). The *Pkib* gene is highly expressed in the testes, and was predicted thought to have a strong reproductive phenotype. Again, KO of this gene produced a minimal phenotype, with normal reproductive ability in both males and females, either in isolation, or when crossed with the *Pkia* null mice. (Belyamani et al. 2001). *Pkig* has not been knocked out to date.



### AKAP KOs

Given the recent interest in the role of PKA localization, it is perhaps not surprising that the roles of the AKAPs are now being studied using KO mouse models (Mauban *et al.* 2009). In 2002, *Akap4* was knocked out of mice (Miki *et al.* 2002). This X-linked AKAP is primarily expressed in the fibrous sheath of sperm; not surprisingly, these mice exhibited male infertility due to abnormal motility and structure. In 2005, one of the splice variants of *Akap6* (mAKAP) was ablated by removing an alternatively spliced 5'-exon of the gene, leading to loss of the brain-predominant mAKAP $\alpha$  isoform of the protein (Michel *et al.* 2005). Mice lacking this exon expressed normal mAKAP $\beta$  in the heart, but were small and had early mortality. At the biochemical level, there was a loss of PDK1 localization, with evidence for loss of downstream activation of p90RSK in the mutant animals.

Recent reports have included KO models for *Akap1* (Newhall *et al.* 2006), *Akap5* (Hall *et al.* 2007, Lu *et al.* 2007, Navedo *et al.* 2008, Schnizler *et al.* 2008, Tunquist *et al.* 2008), *Akap8* (Yang *et al.* 2006), and *Akap10* (Tingley *et al.* 2007), and *Akap12*. In each of these reports, ablation of the specific AKAPs has demonstrated important effects on PKA signaling in specific tissues, including findings in oocyte maturation (*Akap1*), cardiac function (*Akap10*), and most prominently in neuronal function (*Akap5*). These studies confirm the importance not only of PKA subunit abundance, but also of proper localization within the cell (Mauban *et al.* 2009).

### Understanding the function of PKA subunits by targeted gene KO models

With use of global gene KO mouse models, the function of each different PKA subunit has been intensively explored in the process of development and physiology, and particularly in tumorigenesis (Brandon *et al.* 1995, Cummings *et al.* 1996, Burton *et al.* 1999, Amieux *et al.* 2002, Rao *et al.* 2004, Veugelers *et al.* 2004, Kirschner *et al.* 2005). However, the utility of these conventional KO mouse models was limited in observing phenotypes due to embryonic or neonatal lethality that prevented analysis of the impact of PKA subunits in postnatal and adult life. The emergence of the Cre-loxP system has overcome this disadvantage. The Cre (Causes recombination) protein of bacteriophage P1 directs recombination between loxP (locus of crossover P1) sites and deletes gene sequences that are flanked by two 34-bp loxP sites (floxed genes; Sauer & Henderson 1988). By using the Cre-loxP system,

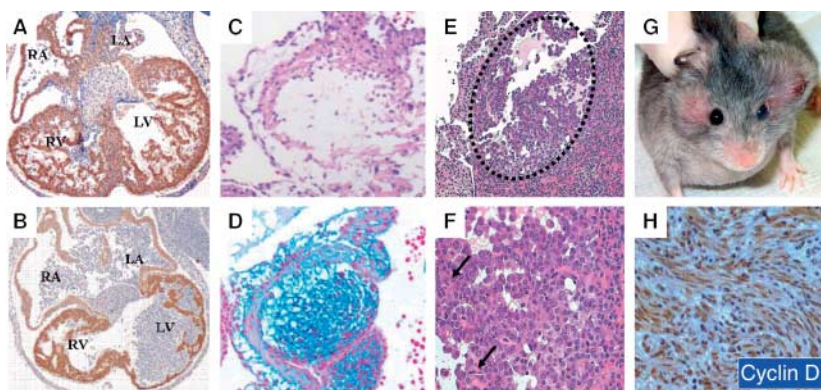
researchers can investigate the role of a target gene at a specific tissue and/or at specific stages of development. Because conventional KO of *Prkar1b*, *Prkar2a*, or *Prkar2b* is viable and fertile (see above), it has not been necessary to pursue a conditional KO strategy for these genes. However, conditional KO of the *Prkar1a* gene has proven a valuable model for understanding the relationship of mutation of this gene in tumorigenesis (Jones *et al.* 2008, Yin *et al.* 2008a,b). The *Prkaca* gene KOs also exhibit significant morbidity and early mortality; however, conditional KO alleles for this gene have not yet been described.

### Tissue-specific R subunit mouse modeling

#### *Heart-specific ablation of Prkar1a causes failure of heart development and myxomagenesis*

As described above, mutations of *PRKARIA* with consequent dysregulation of PKA activity are the cause of CNC in at least 50% of cases (Kirschner *et al.* 2000a,b) or even higher at 65% from a recent analysis of a cohort of 51 CNC probands (Veugelers *et al.* 2004), in which cardiac myxomas presents as one of its cardinal features. Approximately 30–60% of CNC patients will develop cardiac myxomas (Bertherat 2006), and cardiac myxomas are responsible for the death of more than 50% of patients (Stratakis *et al.* 2001). These tumors cannot be recapitulated by conventional *Prkar1a* KO mice because *Prkar1a*<sup>-/-</sup> mice die by e9.5 without the development of heart tube (Amieux *et al.* 2002, Kirschner *et al.* 2005), whereas heterozygotes do not exhibit a cardiac phenotype (Veugelers *et al.* 2004, Kirschner *et al.* 2005). In order to better understand the tumorigenesis of cardiac myxoma, we generated heart-specific *Prkar1a* KO mouse model (*Prkar1a*-CKO) by utilizing the Cre-loxP system (Yin *et al.* 2008a,b), in which  $\alpha$ -myosin heavy chain ( $\alpha$ -MHC) was used as the promoter to drive the expression of cre recombinase.

The *Prkar1a*-CKOs exhibited marked thinning of ventricular walls, characterized by both hypoplasia of the compact layer and severely reduced trabeculation, and died embryonically at e11.5 (Fig. 2A and B). Strikingly, the *Prkar1a*-CKO hearts exhibited disorganization of the sarcomeres by ultrastructural analysis of cardiomyocyte architecture at 11.5. Z disks were easily seen, but they were not properly assembled, and myofilaments appeared loose and disarrayed. Most likely, the disorganized Z disks and myofilaments contribute to poor cardiac contraction and heart failure, causing demise of the growing embryo.



**Figure 2** Pathology in tissue-specific *Prkar1a* knockouts. (A) Normal mouse heart at e11.5 stained for myosin to identify cardiomyocytes. (B) Dilated heart in cardiac-specific *Prkar1a* KO. (C and D) Myxomatous changes in the septa of *Prkar1a*-CKO heart, shown with H and E staining (C) or by Alcian blue staining for extracellular matrix (D). (E) Pituitary microadenoma in a *Prkar1a*-PitKO mouse. (F) Higher power photomicrograph of the tumor showing mitotic figures (arrows). (G) Gross appearance of tumors in a TEC3KO mouse. (H) Cyclin D1 staining of a schwannoma showing overexpression of this protein. See text for details of each of these models.

At the cellular level, the thinning of ventricular walls was caused by the reduction of cardiomyocyte proliferation. In concordance with the reduced levels of the R1a regulatory subunit, hearts from *Prkar1a*-CKO mice exhibited enhancement of both free and total PKA activity, similar to the observation in *Prkar1a*<sup>-/-</sup> mouse embryonic fibroblasts *in vitro* (Nadella & Kirschner 2005). However, ablation of *Prkar1a* did not cause compensation by the other regulatory subunits.

In *Prkar1a*-CKO hearts, there was significant down-regulation of all cardiac transcription factors investigated, including *Srf*, *Nkx2-5*, *Gata4*, and myocardin (*Myocd*), and also downstream targets, such as the cardiac structural proteins  $\beta$ -MHC and actins A1 (*Acta1*) and A2 (*Acta2*). The defective expression of *Acta1* and *Acta2* likely underlies the ultrastructural defect of sarcomeres. These effects of reduction in multiple transcription factors may be due to a direct effect of excess PKA activity on some gene transcription factors (via CREB) or may be an indirect effect on other transcription factors because PKA has previously been demonstrated to interfere with *Srf* activity (Davis *et al.* 2003, Nguyen *et al.* 2004). In addition, *Srf* can interact with *Nkx2-5*, GATA factors and *Myocd* to synergistically activate various transcriptional target genes (Bruneau 2002). The fact that transcription factors function in combination and in hierarchical networks (Durocher *et al.* 1997, Bruneau 2002) may explain why a reduction in multiple proteins, as observed here, causes synergistic and deleterious effects during cardiogenesis.

Very interestingly, ~50% of *Prkar1a*-CKO hearts displayed stroma-rich lesions suggestive of human cardiac myxomas (Fig. 2C and D). At dissection of

e11.5 mice, typical myxoid material was frequently noted in the endocardial cushion. This structure is composed of a subset of cells that undergo remodeling to form the valvular structures and membranous septa of the mature heart after cardiac looping (Person *et al.* 2005). This observation may help explain the observation that myxomas arise predominantly in the interatrial septum (Reynen 1995). With regard to the histogenesis of cardiac myxomas, this finding supports the hypothesis that cardiac myxomas originate from primitive pluripotent mesenchymal cells (Kinsella & Fitzharris 1980, Person *et al.* 2005). Histologically, the majority of *Prkar1a*-CKO hearts exhibited the characteristic features of cardiac myxomas, including the presence of small numbers of polygonal or stellate cells surrounded by abundant loose stroma rich in acid mucopolysaccharides. Most strikingly, they also exhibit epithelium- or gland-like changes, which are rare but diagnostic features of human cardiac myxomas (Goldman *et al.* 1987, Johansson 1989). Finally, the tumors showed evidence of nuclear polymorphism and cellular atypia, as is typically seen only in neoplastic cells. Thus, the evidence is convincing that this mouse model develops early phases in the development of cardiac myxoma.

Although *Prkar1a*-CKO mice did not survive long enough to develop bona fide myxomas, they may still serve as the first good model with which to study the formation of cardiac myxomas. By applying an inducible Cre mouse line strategy, cardiac myxomas in close analogy to human patients may be generated in the future.

### *Pituitary-specific ablation of Prkar1a causes pituitary tumorigenesis*

Pituitary tumors are fairly common neoplasms in the population. A recent meta-analysis estimated the prevalence rate of pituitary adenomas to be ~17% (Ezzat *et al.* 2004). Given the high frequency of pituitary adenomas and their potentially serious effects, the early diagnosis and treatment is very important for patients. However, little is known about the precise environmental and genetic factors involved in pituitary tumorigenesis. In CNC, GH-producing pituitary adenomas are observed in ~10% of patients (Stratakis *et al.* 2001), and prolactinomas have also been reported in CNC kindreds (Stergiopoulos *et al.* 2004). In both of the *Prkar1a*<sup>+/-</sup> mouse models described (Veugelaers *et al.* 2004, Kirschner *et al.* 2005), pituitary pathology was not observed. Therefore, it was hypothesized that haploinsufficiency of *Prkar1a* might not be sufficient to promote tumorigenesis and that complete loss of *Prkar1a* is necessary for tumor formation in the pituitary. To confirm this hypothesis, we used the rat GHRH receptor promoter to generate mice, which express the cre recombinase in pituitary cells of the Pit-1 lineage, which includes cells that produce GH, prolactin (PRL), and TSH (Yin *et al.* 2008a,b). These mice were then crossed with *Prkar1a* conditional null animals (Kirschner *et al.* 2005) to produce pituitary-specific KO (*Prkar1a*-pitKO) mice (Yin *et al.* 2008a,b).

In contrast to the studies of *Prkar1a*<sup>+/-</sup> mice, the *Prkar1a*-pitKOs developed pituitary tumors with high frequency (48%) compared with controls (18%;  $P < 0.05$ ), although not until the advanced age of 18 months (Fig. 2E and F). Given the fact that it took the mice an extended period of time to develop tumors, it is possible that loss of *Prkar1a* alone is not sufficient for tumorigenesis, and that a second genetic hit is required for tumor formation. Although prolactinomas were observed both in control and *Prkar1a*-PitKO mice, non-prolactinomas exhibited much higher incidence rate in the *Prkar1a*-pitKOs (29%) compared with the controls (4%,  $P < 0.05$ ). Characterization of these tumors demonstrated they were composed of cells of the Pit1 lineage that stained for GH, PRL, and TSH.

Interestingly, as is seen in CNC patients, *Prkar1a*-pitKO animals exhibited markedly elevated GH levels in serum compared with controls ( $P < 0.01$ ), regardless of the presence of frank tumors. This may be of particular relevance given that there is a substantial portion of human patients with acromegaly that are not surgically cured of their disease. This model may therefore serve as a good model not only for the

pituitary tumorigenesis but also as a model to test new therapies aimed at acromegaly in human patients.

### *Tissue-specific ablation of Prkar1a in neural crest causes Schwannomas*

Schwannomas are observed in ~14% of CNC patients (Stratakis *et al.* 2001) and have been designated as psammomatous melanotic schwannomas for their histopathologic appearance and high degree of pigmentation (Carney 1990, Martin-Reay *et al.* 1991).

In a *Prkar1a*<sup>+/-</sup> mouse model, schwannomas were observed in ~33% of animals, and facial neural crest-specific KO of the *Prkar1a* gene recapitulated Schwann cell tumorigenesis (Kirschner *et al.* 2005). However, the role of *Prkar1a* ablation on the pathways leading to Schwann cell tumorigenesis was unclear. To address this question, the TEC3 line was developed to express cre under the control of the enhancer-less *Tyrosinase* promoter, which enables transgene expression in a limited subset of facial neural crest derivatives (Tonks *et al.* 2003). This cre line was then crossed with mice carrying a conditional null allele of *Prkar1a* (Kirschner *et al.* 2005) to generate tissue-specific KO mice (*TEC3*; *Prkar1a*<sup>loxP/loxP</sup>, henceforth called TEC3KO animals).

Observation of a cohort of 35 animals revealed that the penetrance of the tumor phenotype was ~50% by 18 weeks and nearly 80% by 40 weeks (Fig. 2G and H; Jones *et al.* 2008). Histopathologically, these heterogeneous neoplasms were clinically classified as Genetically Engineered Mouse Schwannoma, grades II and III (Stemmer-Rachamimov *et al.* 2004).

Because schwannomas are also a cardinal feature of the inherited neurofibromatosis (NF) tumor syndromes, we studied the proteins responsible for both NF1 (Neurofibromin) and NF2 (Merlin) in *Prkar1a*-associated tumors. In TEC3KO schwannomas, the most striking observation was the marked down-regulation of both Neurofibromin and Merlin in the tumors, shown both by immunofluorescence and by Western blot analysis, despite the fact that transcript levels of the *NF1* and *NF2* genes were increased, implying post-transcriptional regulation. It was previously reported that there are interactions between PKA and the NF proteins, as PKA is known to phosphorylate neurofibromin (Izawa *et al.* 1996, Tokuo *et al.* 2001), and loss of neurofibromin up-regulate cAMP levels, which would presumably increase PKA activity (Kim *et al.* 2001). There is also data indicating that PKA-mediated phosphorylation of the NF2 protein Merlin can affect its tumor suppressor function. (Alfthan *et al.* 2004, Laulajainen *et al.* 2008) function. We also observed down-regulation of Merlin



in TEC3KO tumors (Jones *et al.* 2008), although the mechanism remains under investigation. These findings suggest that PKA may play a role in regulating the function and stability of the NF proteins.

Surprisingly, this unique model of Schwann cell tumorigenesis occurs in the absence of Ras (and therefore extracellular signal-regulated kinase, ERK or Akt) pathway activation, in contrast to Schwannomas caused by the ablation of neurofibromin. In the latter, loss of neurofibromin causes up-regulation of Ras signaling, thereby leading to activation of downstream effectors such as ERK and Akt (Cichowski *et al.* 2003, Dasgupta & Gutmann 2005, Johannessen *et al.* 2005, 2008, Guilding *et al.* 2007). These results mimic earlier findings from our laboratory in *Prkar1a* KO MEFs, which also showed that immortalization occurred independently of ERK or Akt activation (Nadella & Kirschner 2005). Furthermore, in TEC3KO schwannomas, only Rac1 exhibited significant activation among the small G proteins of Rho family, which is known to be involved in NF signaling (Pelton *et al.* 1998). There is good evidence that PKA can specifically affect the activity of Rac and Rho in cell type- and condition-specific pattern (Howe 2004). In particular, PKA directly phosphorylates Rho at S188 and negatively regulates its activity (Ellerbroek *et al.* 2003), whereas Rac lacks a direct PKA phosphorylation site. Nevertheless, the effects of the activation of Rho and Rac on the pathway of TEC3KO tumorigenesis need to be further explored.

In summary, PKA may have multiple roles in promoting Schwann cell tumorigenesis. First, it seems to modulate the stability of the NF proteins, such that dysregulation of PKA leads to post-transcriptional loss of these proteins during tumorigenesis. Secondly, PKA seems to signal, either directly or indirectly, to the same downstream effectors activated by mutations in the NF genes. However, unlike activation of Schwann cell tumorigenesis by NF mutations, PKA seems to promote tumorigenesis by a mechanism that excludes activation of Ras, ERK, and Akt. Thus, PKA promotes Schwann cell tumors by mechanisms that overlap but are distinct from those seen in NF associated tumorigenesis.

## Transgenic approaches to studying PKA signaling in the mouse

### Generalized transgenics

Because of the well-known association between activated G-proteins (such as in the MAS) and PKA signaling, efforts have been made to generate a transgenic mouse carrying an activated *Gnas1* gene.

Similar to the observations from human patients, mice carrying activated *Gnas1* in all tissues do not survive gestation (Lee Weinstein, personal communication). This observation speaks to the requirement for control of PKA signaling during embryogenesis.

As a means to analyze the effect of loss of the *Prkar1a* gene, Stratakis *et al.* generated a mouse containing an anti-sense transgene targeted to the *Prkar1a* gene (Griffin *et al.* 2004a,b). This was placed under the control of a doxycycline-suppressible promoter to circumvent any problems with embryonic lethality. Despite the fact that *Prkar1a* null mice exhibit embryonic lethality (Amieux *et al.* 2002, Kirschner *et al.* 2005), the *Prkar1a*-antisense mice exhibited normal development. As they aged, the mice developed a variety of neoplastic lesions, including lymphomas, thyroid follicular adenomas, and mesenchymal tumors. Although the mouse demonstrated enhanced PKA activity (Griffin *et al.* 2004a,b), the connection between this observation and tumorigenesis was not completely clear, due to the potential presence of non-specific effects mediated by the anti-sense mRNA.

### Transgenic approaches targeted to specific tissues

#### Thyroid-specific transgenic models

Determination of the role of PKA signaling in the mouse thyroid has been a topic that has been studied extensively. There is significant interest in understanding the connection between PKA signaling and human thyroid cancer because of recent studies indicating an increased risk of thyroid cancer associated with elevated TSH levels (Haymart *et al.* 2008a,b). The classic approach to activating PKA signaling in the mouse thyroid relies on treatment of mice with anti-thyroidal agents, thus raising TSH and driving PKA signaling. This process is more complex, though, as the TSH-R may signal through multiple G-protein pathways, including Gs, Gi, and Gq (Postiglione *et al.* 2002).

Transgenic approaches to studying PKA signaling by genetically manipulated mouse models have included various means of activating PKA signaling directly in the thyroid. To date, there are three genetic models that have studied the activation of the PKA signaling pathway in the thyroid. The earliest of these was the generation of transgenic mice carrying the A2-adenosine receptor in the thyroid gland by using the bovine thyroglobulin (*bTg*) promoter to drive its expression (Ledent *et al.* 1992). This receptor signals essentially exclusively through Gs, and were generated to mimic constitutive signaling through the TSHR. These mice became significantly hyperthyroid with



large thyroid goiters, and the mice died around 9 months of age from heart failure as a result of their severe hyperthyroidism. Although the glands were hyperplastic, no evidence of thyroid malignancy was observed, even when mice were treated with anti-thyroidal agents to prolong their lifespan.

Next, a transgenic line was generated by introducing a constitutively active mutant of the alpha subunit of the stimulatory G protein (*GNAS*<sup>R201H</sup>) into the thyroid by means of the same *bTg* promoter (Michiels *et al.* 1994). The mutation, which is one of the mutations causing constitutive activation of the GNAS signaling, in MAS, causes unregulated production of cAMP. Despite their hyperthyroidism, these mice survived better than the *bTG-A2R* transgenics, and the thyroid was studied up to 16 months of age. Overall, the thyroid phenotype was again similar, with toxic goiters developing, but no cancers. Finally, the most recent model, still dating back over 10 years ago, was the generation of mice carrying the Cholera toxin (CT) A1 subunit under the control of the rat Tg promoter (Zeiger *et al.* 1997). This CT-A1 subunit causes irreversible activation of Gs by catalyzing its ADP-ribosylation. Three Tg-CT lines were developed, with varying copy numbers of the transgene, leading to varying levels of cAMP production. In each of these lines, the mice eventually developed hyperthyroidism and hypertrophy, but again, no cancers. Additionally, a similar phenotype can be produced pharmacologically by the use of the anti-thyroid agents. In this case, the drug is given to make the mice profoundly hypothyroid, in which they develop marked TSH elevation and thyroid goiters. This model is considered a model of excess TSH stimulation, although unlike the models described above, the mice are hypothyroid, rather than hyperthyroid. When this treatment has been carried out for extended periods, thyroid cancers have not been observed (Jemec 1977).

#### Heart-specific transgenic models

Studies of PKA action in the heart have identified both positive and negative roles of PKA signaling in normal cardiac function. In the heart, cAMP production is classically mediated by the  $\beta$ 1- and  $\beta$ 2-adrenergic receptors (Lohse & Engelhardt 2001), although other receptors also utilize this same second messenger system (Mendez & LaPointe 2005). PKA has multiple well-defined target proteins in the heart, including key functional proteins such as cardiac Troponin I (cTnI), the Ryanodine receptor 2 (Ryr2) and the sarcoplasmic  $\text{Ca}^{++}$ -ATPase. These effects modulate the actions of catecholamines on cardiac function. Up-regulation

of PKA activity has been posited to occur in the failing heart, as there is increased phosphorylation of PKA targets (Marx & Marks 2002, Reiken *et al.* 2003), providing an explanation for the beneficial effects of  $\beta$ -blockade in these patients.

From a genetic point of view, dysregulation of the PKA system has been associated with abnormalities of heart function in mouse models (Lohse & Engelhardt 2001), although corresponding human syndromes are generally unknown. Specific studies using transgenic mice to target the heart include the use of WT or mutant  $\beta$ -adrenergic receptors, present either at normal levels or overexpressed and thus constitutively active. In general, these studies have shown that chronic overexpression of the  $\beta$ 1-adrenergic receptors leads to cardiomyopathy and eventual heart failure (Engelhardt *et al.* 1999, Bisognano *et al.* 2000). This same phenotype was observed with expression of activated *Gnas1* (Vatner *et al.* 1998) or of the  $\beta$ -adrenergic receptor kinase (Koch *et al.* 1995), confirming the detrimental effects on chronic stimulation of this pathway. The data for the  $\beta$ 2-adrenergic receptor are a little more complicated, with moderate level expression of this receptor being tolerated, but high level expression causing significant cardiomyopathy (Liggett *et al.* 2000).

However, the situation is not quite so straightforward, as shown in studies with the type VI AC. When this protein was overexpressed in mice with cardiomyopathy-induced by overexpression of  $\text{G}\alpha\text{-q}$  enhancement of cyclase increased survival, possibly by a mechanism that involves enhanced response to catecholamines (Roth *et al.* 1999, Rebollo *et al.* 2006). However, transgenic expression of the same gene did not cause significant effects on heart rate or basal function (Roth *et al.* 2003), although it did improve survival in a coronary artery ligation model (Takahashi *et al.* 2006).

In a direct model, the PKAC subunit was introduced into the heart under the control of the  $\alpha$ -MHC promoter (Antos *et al.* 2001). Similar to the observations in the *Prkar1a* cardiac KO animals (Yin *et al.* 2008a,b), the mice developed dilated cardiomyopathy which correlated with the degree of transgene expression. These mice exhibited enhanced phosphorylation of the Ryr2 and the calcium handling protein phospholamban, in accordance with prior studies of heart failure.

An additional approach that has recently been studied is the effect of disrupting PKA localization in the heart. In this approach, a fragment of the AKAP AKAP13/AKAP-Lbc, termed Ht-31 (Vijayaraghavan *et al.* 1997), is overexpressed in the cell. Owing to the competition for binding to PKA, primarily via the type 2 regulatory

subunits, subcellular localization is disrupted. When Ht-31 was expressed under an adenoviral control system, minimal effects were observed at baseline; however, stimulation with high doses of adrenergic agonist led to reductions in phosphorylation of cTnI and caused enhanced  $\beta$ -adrenergic dependent cardiac contractility (McConnell *et al.* 2009).

An alternative approach to studying PKA signaling has been the use of a mutated form of the CREB transcription factor, which lacks the site for PKA (and other kinase) phosphorylation sites at S133. Targeting expression of this protein to the heart also produced dilated cardiomyopathy and electrophysiological changes (Fentzke *et al.* 1998, Zhu & Saba 2003), again highlighting that either over- or under-activation of the PKA pathway produces deleterious changes.

#### Brain-specific transgenic models

One of the striking observations from PKAR subunit conventional KO models has been the general subtlety of the phenotypes. After careful investigation, CNS effects were observed in KO models for R1b (Brandon *et al.* 1995, Huang *et al.* 1995), R2a (Rao *et al.* 2004), and R2b (Brandon *et al.* 1998), as well as catalytic subunit KOs (Huang *et al.* 1995, Qi *et al.* 1996, Howe *et al.* 2002).

These observations parallel closely an extensive literature regarding the effects of PKA dysregulation in the CNS. The importance of PKA function for proper cognition in the mouse dates back to the late 90's when Abel *et al.* generated mice with a dominant negative form of the PKAR1 subunit known as R(AB), which has mutations in the cAMP-binding sites (Clegg *et al.* 1987). Transgenic mice expressing this cDNA under the control of the brain-directed *CAMKII* promoter exhibited defects in LTP, and important component of long-term memory (Abel *et al.* 1997). This system has formed the basis for extensive investigation on the genetics and biochemistry of memory associated with disruption of PKA, either by genetic means or by pharmacologic measures in isolated brain slices. This field has been recently reviewed extensively (Abel & Nguyen 2008), and will not be discussed in detail, other than to note that all studies indicate the importance of proper PKA regulation in the establishment and maintenance of long-term memory, not only in mice, but in lower organisms as well.

#### Neural crest models

The McKnight group recently reported the development of a cre-inducible form of the R(AB) dominant negative *Prkar1a* protein. In this model, the expression of R(AB) is blocked by a translational stop codon that

is excised by cre-mediated recombination. When mice carrying this construct were crossed with mice expressing cre in the neural crest, the progeny exhibited lethal intestinal pseudo-obstruction, which was traced to a defect in the enteric ganglia (Howe *et al.* 2006). Although this allele of a dominant negative PKA has not yet been used extensively, it will provide another tool for further dissection PKA signaling pathways.

## Summary and future directions

The cAMP-PKA signaling system has been known for over 40 years, and extensive work has gone into characterizing how stimulation or inhibition of this pathway affects cell function. Until recently, most of these studies were carried out in the context of immortalized tissue culture cells. Aside from problems associated with the presence of multiple genetic mutations that enable these cells to survive in culture, the use of a homogenous cell population in which intercellular communication is of minimal importance may undervalue the true role of this system, which appears to be involved in intercellular signaling in most tissues.

For this reason, the use of mouse models, which enable the assessment of genetic changes in the setting of an entire organism, allows a much better appreciation of the importance of this signaling system. The use of PKA subunit KOs has revealed the essential role of the *Prkar1a* regulatory subunit and the near-essential role of the *Prkaca* catalytic subunit. The role of the other subunits in physiologic processes is much more subtle, with the exception of the *Prkar2b* subunit, which clearly has major roles in regulating both CNS function and fat metabolism.

As the study of PKA action moves forward, the tools are now in place to assess how these alterations affect signaling at the tissue level. Despite the fact that mice lacking *Prkar1a* in Schwann cells develop tumorigenesis, this occurs independently of pathways thought to be important *in vivo* for tumor formation. As the signaling pathways affected by each of these KOs is traced at the molecular level, we should expect to gain important insights into direct signaling mechanisms, as well as how these pathways participate in important cross talk interactions.

Although this knowledge will be important for its own sake, the ultimate goal of these investigations will be to use the information for therapeutic purposes. Phosphodiesterase inhibitors, which prolong the cAMP-dependent signal, are already in use in clinical practice (sildenafil, tadalafil, etc.) and others have been tried (rolipram). Although disappointing so far, the study of the CNS functional alterations may lead to

new therapies for disorders such as depression or for movement disorders. As we better understand how dysregulation of PKA causes tumors in the CNC, in the thyroid, and in other organs (e.g. pituitary) this same approach may be used to affect tumor behavior and thereby lessen the impact of these diseases.

The availability of mouse models for these studies marks an important advance, which may lead to new developments in many of these fields. Stay tuned...

## Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

## Funding

This work was supported by the National Institute of Child Health and Human Development (grant number HD01323), the National Cancer Institute (grant numbers CA112268 and CA16058), the US Army Medical Research and Materiel Command (grant number NF043067), and the Children's Tumor Foundation (grant number 2006-01-026).

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