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Either *Kras* activation or *Pten* loss similarly enhance the dominant-stable CTNNB1-induced genetic program to promote granulosa cell tumor development in the ovary and testis

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Abstract

WNT, PI3K or RAS signaling pathways control specific stages of ovarian follicular development. To analyze the functional interactions of these pathways in granulosa cells during follicular development *in vivo*, we generated specific mutant mouse models. Stable activation of the WNT signaling effector beta-catenin (CTNNB1) in granulosa cells results in the formation of premalignant lesions that develop into granulosa cell tumors (GCTs) spontaneously later in life or following targeted deletion of the tumor suppressor gene *Pten*. Conversely, expression of oncogenic $KRAS^{G12D}$ dramatically arrests proliferation, differentiation and apoptosis in granulosa cells, and consequently, small abnormal follicle-like structures devoid of oocytes accumulate in the ovary. Because of the potent anti-proliferative effects of $KRAS^{G12D}$ in granulosa cells, we sought to determine if $KRAS^{G12D}$ would block precancerous lesion and tumor formation in follicles of the CTNNB1 mutant mice. Unexpectedly, transgenic *Ctnnb1;Kras* mutant mice exhibited increased GC proliferation, decreased apoptosis and impaired differentiation and developed early-onset GCTs leading to premature death in a manner similar to the *Ctnnb1;Pten* mutant mice. Microarray and RT-PCR analyses revealed that gene regulatory processes induced by CTNNB1 were mostly enhanced by either KRAS activation or *Pten* loss in remarkably similar patterns and degree. The concomitant activation of CTNNB1 and KRAS in Sertoli cells also caused testicular granulosa cell tumors that showed gene expression patterns that partially overlapped those observed in GCTs of the ovary. Although the mutations analyzed herein have not yet been linked to adult GCTs in humans, 1) other components of these pathways may be altered or mutated, 2) these mutations may relate to juvenile GCTs or 3) they may occur in tumors of other tissues where CTNNB1 is mutated. Importantly, our results provide strong evidence that CTNNB1 is the driver in these contexts and that $KRAS^{G12D}$ and *Pten* loss promote the program set in motion by the CTNNB1.

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Keywords

CTNNB1; KRAS; PTEN; granulosa cell tumor; ovary; testis

Introduction

Granulosa cells are the most proliferative cell type in the ovary (Rao MC, Midgley AR *et al.*, 1978; Robison GAR, Butcher RW *et al.*, 1971). Because of this it is surprising that granulosa cell tumors (GCTs; classified among sex-cord/stromal neoplasms) are relatively rare in mammals. This may be determined by apoptosis which is a potent mechanism for eliminating granulosa cells in follicles that fail to differentiate or by luteinization which terminates granulosa cell proliferation (Tilly JL, Tilly KI *et al.*, 1995) and leads to the non-dividing luteal phenotype (Rieske P Pongubala JMR, 2001). Despite their rarity, GCTs remain the most common ovarian cancer subtype in most domestic species. For reasons that are not completely understood, women are particularly prone to developing cancers arising from the ovarian surface epithelium, and for this reason GCTs only represent around 5% of all human ovarian cancer (Jubb I, Kennedy KVF *et al.*, 1993; Schumer ST Cannistra SA, 2003). GCTs can also occur in the testis (Dilworth JP, 1991). Despite the impact of GCTs on domestic species and humans, the molecular mechanisms underlying the etiology of this disease are not yet entirely clear, only a limited number of mouse models have been generated (Edson MA, Nalam RL *et al.*, 2010; Matzuk MM, Finegold *et al.*, 1992; Pangas SA, Li X *et al.*, 2008) and their relevance to domestic and human GCTs remains to be clearly determined.

Recent studies indicate that stage-specific activation of the WNT/FZD/CTNNB1/TCF cascade plays critical roles in controlling normal follicular development and granulosa cell tumor formation (Boyer A, Goff AK *et al.*, 2009). If components of canonical WNT pathway are inappropriately activated or disrupted, granulosa cell fate decisions and follicular growth are dramatically altered (Boerboom D, Paquet M *et al.*, 2005; Boerboom D, White LD *et al.*, 2006; Boyer A, Goff AK *et al.*, 2009; Boyer A, Lapointe E *et al.*, 2010; Lague MN, Paquet M *et al.*, 2008; Vainio S, Heikkila M *et al.*, 1999). *Wnt4* null mice provided the first evidence that the WNT/beta-catenin (CTNNB1) canonical pathway exerts potent effects on embryonic gonadal development by suppressing male gonad formation and facilitating ovarian development (Vainio S, Heikkila M *et al.*, 1999). Conversely, expression of a stable, activate, mutant form of CTNNB1 selectively in granulosa cells leads to the formation of precancerous lesions that eventually become granulosa cell tumors (Boerboom D, Paquet M *et al.*, 2005; Boerboom D, White LD *et al.*, 2006). In follicles where granulosa cells escape early transformation but express stable activated CTNNB1, FSH mediated induction of *Cyp19a1* and granulosa cell proliferation are enhanced whereas LH mediated granulosa cell differentiation is completely blocked (Fan HY, O'Connor A *et al.*, 2010). Moreover, FSH and other signaling factors canactivate the WNT signaling cascade leading to transcriptional activation of CTNNB1 (Fan HY, O'Connor A *et al.*, 2010; Parakh TN, Hernadez JA *et al.*, 2006). The WNT/CTNNB1 pathway intersects with multiple signaling cascades and transcription factors to dramatically alter cell function during development and in cancer (Boyer A, Goff AK *et al.*, 2009; Jin T, George Fantus I *et al.*, 2008; Wodarz A

Nusse R, 1998). For example, FOXL2 acts coordinately with WNT4 to regulate formation of the embryonic ovary and impacts granulosa cell proliferation and differentiation in the postnatal ovary (Boyer A, Lapointe E *et al.*, 2010; Jamieson S, Butzow R *et al.*, 2010; Ottolenghi C, Pelosi E *et al.*, 2007; Uda M, Ottolenghi C *et al.*, 2004; Wang HX, Li TY *et al.*, 2010). Moreover, a specific mutation (C134W) in the human *Foxl2* gene has recently been shown to be expressed in nearly all adult GCTs indicating that it plays a critical role in this disease in women (Jamieson S, Butzow R *et al.*, 2010; Kalfa N, Fellous M *et al.*, 2008; Kobel M, Gilks CB *et al.*, 2009; Shah SP, Kobel M *et al.*, 2009).

The RAS pathway is also critical for normal ovarian function (Fan HY Richards JS, 2010). LH induction of the EGF-like factors amphiregulin (AREG), epiregulin (EREG) and betacellulin (BTC) and their activation of the EGF receptor/RAS/ERK1/2 pathway in granulosa cells of preovulatory follicles regulate ovulation, oocyte maturation and the terminal differentiation of granulosa cells to non-dividing luteal cells (Ashkenazi H, Cao X *et al.*, 2005; Conti M, Hsieh M *et al.*, 2005; Fan HY, Liu Z *et al.*, 2009b; Hsieh M Conti M, 2005; Hsieh M, Theologis A *et al.*, 2006; Park J-Y, Su Y-Q *et al.*, 2004). When the KRAS/ERK1/2 pathway is activated selectively in granulosa cells at earlier stages of follicular development by expressing a mutant $KRAS^{G12D}$, the $Kras^{G12D}$ mutant granulosa cells cease dividing and fail to differentiate (Fan HY, Shimada M *et al.*, 2008). Because apoptosis is also blocked, abnormal follicle-like structures persist and accumulate in the ovary leading to premature ovarian failure (Fan HY, Shimada M *et al.*, 2008). Thus, expression of $Kras^{G12D}$ in granulosa cells does not exhibit oncogenic activity, at least in this context. Rather, it causes granulosa cells to exit the cell cycle and this is associated with elevated levels of the tumor suppressor, PTEN (Fan HY, Liu Z *et al.*, 2009a; Fan HY, Shimada M *et al.*, 2008).

Components of the PTEN/PI3K/AKT/FOXO pathway are expressed in granulosa cells and are activated not only by IGF1 but also by FSH, LH and the EGF-like factors (Fan HY, Liu Z *et al.*, 2009b; Gonzalez-Robayna IJ, Falender AE *et al.*, 2000; Richards JS, Sharma SC *et al.*, 2002). Disruption of *Pten* in granulosa cells reduces apoptosis, increases the number of ovulating follicles and the persistence of luteal cell structures (Fan HY, Liu Z *et al.*, 2008). However, the disruption of *Pten* alone rarely leads to GCTs (Lague MN, Paquet M *et al.*, 2008), perhaps because the levels of PTEN are relatively low in normal granulosa cells (Fan HY, Liu Z *et al.*, 2008). Conversely, over-expression of a stable active form of FOXO1, a down-stream target of AKT, severely impairs granulosa cell responses to FSH and LH in culture (Liu Z, Rudd MD *et al.*, 2009).

In the ovary, the proliferative effects of a stable mutant form of *Ctnnb1* in granulosa cells are markedly enhanced if the tumor suppressor *Pten* is deleted coordinately in granulosa cells of the *Ctnnb1* mutant strain leading to rapid tumor growth (Lague MN, Paquet M *et al.*, 2008). Furthermore, when mutations mutations in *Ctnnb1* and *Pten* are directed to Sertolie cells, GCT formation in occurs in the testis (Boyer A, Paquet M *et al.*, 2009), indicating that Sertoli cells are also susceptible to the combined tumorigenic effects of these two oncogenes.

The potent anti-proliferative effects of mutant $KRAS^{G12D}$ in granulosa cells of small follicles appear to be irreversible because proliferation in the $KRAS^{G12D}$ mutant granulosa

cells is not markedly reversed by the loss of the tumor suppressor *Pten* in this context (Fan HY, Liu Z *et al.*, 2009a; Fan HY Richards JS, 2010). These results documented that granulosa cells, unlike ovarian surface epithelial cells (Fan HY, Liu Z *et al.*, 2009a), are highly resistant to these specific oncogenic factors. Therefore, we reasoned that $KRAS^{G12D}$ might block the proliferative and transformation-dependent effects of CTNNB1 in GCTs of the ovary and testis. Therefore, we sought to determine if tumor formation and growth initiated by mutant CTNNB1 could be altered (suppressed) in granulosa cells by co-expressing $KRAS^{G12D}$.

Additionally, we sought to determine if there were cell- and stage-specific effects in response to expressing mutant CTNNB, $KRAS^{G12D}$ or the loss of PTEN. Thus, we have compared gonad morphology, gene expression profiles and functions in mice expressing mutant *Ctnnb1* and *Kras* or mutant *Ctnnb1* and loss of *Pten* driven by mice expressing either *Cyp19Cre* or *Amhr2Cre*. Although these mouse models combine mutations that have not yet been identified in human GCTs, the data obtained provide novel evidence on how these pathways may interact in other tissues where CTNNB1 mutations are common.

Results

Premalignant lesions and GCTs occur in the Ctnnb1;C-Cre mice

Previous studies showed that targeted expression of dominant stable CTNNB1 in granulosa cells using the *Ctnnb1*^{tm1Mmt} (simplified herein to *Ctnnb1*) and *Amhr2*$^{tm3(cre)Bhr}$ (*A-cre*) alleles led to the formation of abnormal follicles characteristic of precancerous lesions (Boerboom D, Paquet M *et al.*, 2005; Boerboom D, White LD *et al.*, 2006). Ultimately, the (*Ctnnb1*$^{tm1Mmt/+}$;*Amhr2*$^{tm3(cre)Bhr/+}$)(*Ctnnb1;A-cre*) mice developed GCTs after 6 months of age. Because *Amhr2Cre* is expressed early during embryonic ovarian development, it was hypothesized that a clonal population of cells in the developing gonad was vulnerable to effects of oncogenic CTNNB1. To determine if expressing dominant stable CTNNB1 at later stages of follicular development could also impair granulosa cell functions leading to precancerous lesions and GCTs, we used the *Cyp19Cre* (Tg(CYP19A1-cre)1jri) (*C-Cre*) mice in which recombinase activity is first detected approximately at postnatal day 15–20 and is increased in preovulatory follicles (unpublished). The *Ctnnb1*$^{tm1Mmt/+}$; Tg(CYP19A1-cre)1jri (*Ctnnb1;C-Cre*) mice lived for 6–8 months (Figure 1A) but also eventually succumbed to GCTs. The ovaries of these mice exhibited visible precancerous lesions between 4–6 weeks of age (Figure 1C) similar to those observed previously in the *Ctnnb1;A-Cre* mice (Boerboom D, Paquet M *et al.*, 2005; Boerboom D, White LD *et al.*, 2006). All wild-type (WT) littermates remained viable and exhibited normal ovarian morphology at 4, 6 and 8–12 weeks of age (Figures 1A, C).

GCTs form in the Ctnnb1;Kras^{G12D};C-Cre and Ctnnb1;Pten;C-Cre mutant mice

To analyze the specific effects of oncogenic *Kras*^{G12D} versus loss of *Pten* in the *Ctnnb1;C-Cre* mutant mice, the *Kras*$^{tm4Tyj/+}$;*Ctnnb1*$^{tm1Mmt/+}$; Tg(CYP19A1-cre)1jri (***Ctnnb1;Kras;C-Cre***) and *Pten*$^{tm1Hwu/}$tm1Hwu;*Ctnnb1*$^{tm1Mmt/+}$; Tg(CYP19A1-cre)1jri (***Ctnnb1;Pten;C-Cre***) mice were generated and checked twice weekly for evidence of tumor growth (distended abdomen) and viability. Because granulosa cells present in the abnormal follicles of the

Kras;C-Cre mice are non-proliferative (Fan HY, Shimada M *et al.*, 2008), we predicted that *Kras*^{G12D} would suppress the proliferative effects of mutant *Ctnnb1*. Unexpectedly, precancerous lesions appeared in the *Ctnnb1;Kras;C-Cre* mice between 4–5 weeks of age (Figure 1C) and bilateral tumors were observed by 3 months of age (Fig. 1B). All *Ctnnb1;Pten;C-Cre* mice died within 2–3 months of age due to tumor burden (Figure 1A; not shown). Histological analyses showed evidence of precancerous lesions by 3 weeks of age and large GCTs by 6–8 weeks of age (Figure 1C). These results are similar to the *Ctnnb1;Pten; A-Cre* mice that we reported earlier (Lague MN, Paquet M *et al.*, 2008). Although the tumors of the *Ctnnb1;Kras;C-Cre* mice grew more slowly than those of the *Ctnnb1;Pten;C-Cre* mice, they grew faster than those in the *Ctnnb1;C-Cre* mice indicating that the presence of *Kras*^{G12D} enhances tumor growth mediated by CTNNB1 but does so at a slower rate compared to the loss of *Pten*.

Fertility is impaired in the Ctnnb1;Kras;C-Cre and Ctnnb1;Pten;C-Cre mutant mice

We next sought to determine the physiological consequences of either expressing KRAS^{G12D} or disrupting *Pten* in the *Ctnnb1;C-Cre* mutant mice. As shown, serum levels of FSH and LH were elevated in the *Ctnnb1;Kras;C-Cre* and *Ctnnb1;Pten;C-Cre* mutant mice at 6 weeks of age compared to WT controls, whereas serum estradiol and progesterone levels were reduced in the mutants (Figure 2A). Despite elevated gonadotropins, granulosa cell differentiation was blocked as indicated by the loss of granulosa cell specific marker genes (*Fshr*, *Lhcgr*, *Cyp19a1*, *Cyp11a1 Inha*, *Amh*) in ovaries of the *Ctnnb1;Kras;C-Cre* and *Ctnnb1;Pten;C-Cre* mice at 6 weeks (Figure 2B). Granulosa cells of immature mice prior to major tumor formation also failed to respond to exogenous eCG as indicated by the lack of induction of specific granulosa cell marker genes in immature *Ctnnb1;Kras;C-Cre* and *Ctnnb1;Pten;C-Cre* mice treated with eCG to induce follicular development (Figure 2C).

Granulosa cell proliferation is increased and apoptosis is decreased in the GCTs

To determine if proliferation was altered in the *Ctnnb1;Kras;C-Cre* and *Ctnnb1;Pten;C-Cre* granulosa cells compared to WT controls, BrdU uptake and levels of phospho-histone-H3 (pHH3) were examined. To determine if apoptosis was altered TUNEL assays detecting DNA fragmentation and immunostaining for FOXO1, an apoptosis related factor in granulosa cells (Liu Z, Rudd MD *et al.*, 2009) that is absent in the abnormal follicle present in the *Kras;C-Cre* mice (Fan HY, Shimada M *et al.*, 2008) were performed. FOXO1 and DNA fragmentation were absent/reduced in the GCTs of the *Ctnnb1;Kras;C-Cre* and *Ctnnb1;Pten;C-Cre* mutant mice but were detected in growing follicles of WT and *Ctnnb1;C-Cre* ovaries (Figure 3A). Immunolabeling of BrdU and pHH3 was highest in the ovaries (GCTs) of the *Ctnnb1;Pten;C-Cre* mice at 5–6 weeks of age, compared to that observed in the *Ctnnb1;Kras;C-Cre* mutant mice and WT mice Figure 3A and B). Thus, apoptosis is reduced and proliferation is increased in the GCTs of the double mutant mice.

Levels and activation of CTNNB1, PTEN and KRAS pathway components in mutant and WT ovaries

To determine the expression levels of CTNNB1, PTEN and components of the KRAS pathway in the mutant mouse ovaries, ovaries were collected and cell lysates were prepared for Western blot analyses. As shown, levels of CTNNB1 were low in ovaries of immature WT mice compared to ovaries in the *Ctnnb1;C-Cre*, *Ctnnb1;Kras;C-Cre* and *Ctnnb1;Pten;C-Cre* mice (Figure 4). The results also confirmed the expression of the mutant CTNNB1 in the relevant ovaries and that levels of PTEN protein were reduced selectively in the *Ctnnb1;Pten;C-Cre* ovaries. Levels of phosphoERK1/2 and phospho-P90RSK, down stream targets of KRAS were elevated in the *Ctnnb1;Kras;C-Cre* and *Ctnnb1;C-Cre* samples but were low in the *Ctnnb1;Pten;C-Cre* granulosa cells. Of particular note, the levels of phospho-AKT and phospho-GSK3β were elevated in all samples expressing mutant CTNNB1 (Figure 4B).

Gene expression profiles reveal a remarkable similarity among the genes expressed in granulosa cells of each mutant genotype

To determine specific genes regulated by CTNNB1 in granulosa cells *in vivo*, microarray analyses were done using RNA samples prepared from ovaries of 4 week old control, *Ctnnb1;C-Cre*, *Ctnnb1;Kras;C-Cre*, and *Ctnnb1;Pten;C-Cre* mutant mice. *In silico* analyses determined that many genes were up-regulated (619) or down-regulated (203) by expression of dominant stable CTNNB1 alone (Figure 5). Of the 619 up-regulated genes 130 (21%) were specific to CTNNB1 alone, whereas 440 (71%) were up-regulated not only in the *Ctnnb1;Pten;C-Cre* mutant mice but also in the *Ctnnb1;Kras;C-Cre* mutant strain. These included CTNNB1 target genes many of which are positive (*Wnt4, Wnt2, Wnt6, Wnt5b, Wnt16, Tcf7, Tcf12*) as well as negative (*Wif1*, *Notum*, *Nkd1* and *Axin2*) regulators of the canonical WNT pathway (Table 1, Figure 6). Of note, two negative regulators of the WNT/CTNNB1 pathway, *Ndk2* and *Dkk2* were increased in the *Ctnnb1;Kras;C-Cre*, and *Ctnnb1;Pten;C-Cre* mutant cells but not in the *Ctnnb1;C-Cre* mutant cells whereas up-regulation of *Ndk1* and *Dkk4* were selectively up-regulated in the *Ctnnb1;C-Cre* mutant cells. Expression of several genes was verified by real-time RT-PCR and *in situ* hybridization (Figure 6) (Supplemental Figures 1&2). Of the 203 down-regulated genes 22 (11%) were specific for CTNNB1 alone whereas 177 (87%) were also down-regulated in both double mutant strains. Within the latter cluster, many of the genes were either granulosa cell specific (*Fshr, Nr5a2, Esr2, Prkar2b, Fst*) or oocyte specific (*Zp3, Oosp1, Nlrp4, Gdf9, Gpr1*) but also included one tumor suppressor (*Rassf4*) that binds RAS and is associated with RAS-dependent apoptosis (Table 1).

Equally impressive was the high number of genes that were up-regulated in the CTNNB1 expressing cells by either $KRAS^{G12D}$ (2136) or the loss of *Pten* (1977) (Figure 5). Of these, a large number (1362) were up-regulated in both the *Ctnnb1;Pten;C-Cre* (64%) and *Ctnnb1;Kras;C-Cre* (69%) mutant ovaries. These results were unexpected given the markedly different effects of expressing $Kras^{G12D}$ (Fan HY, Shimada M *et al.*, 2008) or disrupting *Pten* alone (Fan HY, Liu Z *et al.*, 2008) or in combination in granulosa cells (Table 1) (Fan HY, Shimada M *et al.*, 2008). A notable number of up-regulated genes were related to RAS signaling including the *Ret* and *Met* proto-oncogenes, *Etv5* a potential

regulator of RET (Tyagi G, Carnes K *et al.*, 2009), Epidermal growth factor receptor pathway substrate 8, *Eps8* (Xu M, Shorts-Cary L *et al.*, 2009), *Rrad*, *Rassf8*, several guanine nucleotides exchange factors (*Arhgef2, 3, 5, 12* and *Rapgef4*, also known as *Epac*) and *Rab* family members (*Rab6*, *20*, *24*) involved in intracellular trafficking. Components of the PI3K pathway (*Pik3c3*, *Pik3r3*) were increased ~3-fold and protein kinase C, beta (*Prkcb*) was elevated ~30-fold (Table 1). The parallel up-regulation of components of the RET/MET/RAS and PI3K pathways by $KRAS^{G12D}$ or loss of *Pten* underscores the overlapping impact of these mutations and indicates that these two pathways are tightly linked in these cancer cells.

An impressive number of genes were also down-regulated and showed extensive overlap between in the *Ctnnb1;Kras;C-Cre* (83%) and *Ctnnb1;Pten;C-Cre* (82%) mutant ovaries. Most genes down-regulated in this cluster reflected the loss of granulosa cell markers, including several components of the IGF1 pathway (*Igf1, Igf1r, Irs2, Socs2, Foxo1*) and the inhibin/activin/*Bmpr1b* pathway. Because the mutant ovaries lack corpora lutea and are essentially devoid of oocytes, markers of these cell types were also markedly reduced (Table 1). However, three of the most highly down-regulated genes were osteoglycin (*Ogn*) that encodes a blocker of metastasis, leucocyte cell derived chemotaxin (*Lect1*) that encodes a potent blocker of angiogenesis and *Uchl1* that encodes a ubiquitin carboxy-terminal hydrolase that is associated with cancer progression in a cell context specific manner (Fellenberg J, Lehner B *et al.*, 2010; Hussain S, Foreman O *et al.*, 2010). Other genes of particular interest that were increased in the tumors include *Apcdd1*, *Enpp2*, *Etv1*, *Fst*, *Cnr1*, *Sox7* and *Tbx3* (Table 1, Figure 6).

Ctnnb1;Kras;A-Cre mice develop granulosa cell tumors of the testis

As male *Ctnnb1;Pten;A-Cre* male mice develop granulosa cell tumors of the testis (GCTT) (Boyer A, Paquet M *et al.*, 2009), we next sought to determine if activated KRAS and dominant-stable CTNNB1 could combine to cause GCTT in the *Ctnnb1;Kras;A-Cre* model. Male *Ctnnb1;A-Cre* mice developed a seminiferous tubule degeneration phenotype by 5 weeks of age, resulting in progressive loss of spermatogenesis, testicular atrophy with reduced testis size and sterility (Figure 7A, B, D; Table 2), which was consistent with previous reports (Boyer A, Hermo L *et al.*, 2008; Tanwar PS, Zhang L *et al.*, 2010). Whereas male *Kras;A-Cre* mice were phenotypically normal and had grossly unaltered fertility and spermatogenesis (Fig 7A–C, Table 2 and data not shown), *Ctnnb1;Kras;A-Cre* mice had a degenerative phenotype similar to that observed in the *Ctnnb1;A-Cre* model, but with a somewhat earlier onset (~ 4wks). Furthermore, GCTT that were histologically indistinguishable from those observed in the *Ctnnb1;Pten;A-Cre* model were found in most *Ctnnb1;Kras;A-Cre* testes after 4–5 months of age (Fig 7A, E, F). GCTTs in *Ctnnb1;Kras;A-Cre* mice grew more slowly than those of *Ctnnb1;Pten;A-Cre* mice, resulting in testis/tumor weights at 4–6 months of age that were similar to those of *Ctnnb1;Pten;A-Cre* mice at 5 weeks of age (Fig 7A&B and Table 2). The tumors did not spread or metastasize, and did not compromise viability or animal well-being up to 8 months of age (not shown). The diagnosis of GCTT in the *Ctnnb1;Kras;A-Cre* testes was supported by the detection of ectopic expression of FOXL2 (Fig 7G) and *Wnt4* (Fig 8), both markers of early granulosa cell differentiation (Ottolenghi C, Pelosi E *et al.*, 2007). FOXL2 expression

was in fact comparable to levels expressed in ovarian tissue, as had been previously observed in the *Ctnnb1;Pten;A-Cre* model.

Granulosa cell tumors of the ovary and testis express some similar and some distinct CTNNB1 target genes

Because GCTs develop in the ovaries and testes of the *Ctnnb1;Pten;A-Cre* mutant mouse strain (Boerboom D, Paquet M *et al.*, 2005; Boerboom D, White LD *et al.*, 2006; Boyer A, Goff AK *et al.*, 2009; Boyer A, Paquet M *et al.*, 2009; Lague MN, Paquet M *et al.*, 2008), we used these mice to determine if the tumors in the ovary and testis express similar or distinct genes and if expression of $Kras^{G12D}$ would impact the growth of GCTs in the *Amhr2-Cre* expressing mouse strains. Ovaries and testes were collected from control, *Ctnnb1;A-Cre, Kras;A-Cre, Pten;A-Cre, Ctnnb1;Pten;A-Cre* and *Ctnnb1;Kras;A-Cre* mutant mice at distinct ages when tumor sizes were similar (based on published and preliminary studies). Ovarian and testis weights were determined (Table 2) and the tissues used to prepare RNA. Real time RT-PCR was used to determine the expression of selected genes that were prominently regulated on the microarrays and in tumors of the *C-Cre* mutant strains (Figures 5 and 6). As shown, many CTNNB1 target genes were slightly or significantly up-regulated in ovaries of the *Ctnnb1;A-Cre* mice and were dramatically increased further when either the *Pten* gene was depleted or $Kras^{G12D}$ was expressed in the presence of mutant CTNNB1 (Figure 8). These results confirm those obtained in the *C-Cre* strains. Two genes (*Nr5a2, Nr0b1*) were reduced in ovaries of the double mutant mice and three (*Foxo3a, Tcf12* and *Wnt4*) showed no change. When the same genes were analyzed in the testis samples, several CTNNB1 target genes (*Axin2, Enpp2, Etv1, Nfat5, Ndk1, Tcf12* and *Wnt4*) were increased in the *Ctnnb1;A-Cre* alone mutant testes. Several of these genes were also increased in the *Ctnnb1;Pten;A-Cre* mice whereas expression of *Axin2, Ndk1* and *Peg3* increased most dramatically in the *Ctnnb1; Kras;A-Cre* mutant mice. The increased expression of *Foxo3* and *Wnt4* and the lack of expression of *Cd83* and *Wnt16* in testes of the *Ctnnb1;A-Cre* mice were notably different from the expression patterns of these genes observed in the ovaries of mice of the same genotype.

Discussion

These studies document for the first time that oncogenic $KRAS^{G12D}$ as well as loss of *Pten* promote the altered genetic program set in motion by dominant stable CTNNB1 that drives granulosa proliferation and cell fate decisions (Boerboom D, White LD *et al.*, 2006; Boyer A, Paquet M *et al.*, 2009). Most impressive and unexpected were the phenotypic data showing that oncogenic $KRAS^{G12D}$ and loss *Pten* exert remarkably similar effects in magnitude and direction, leading to early granulosa cell tumor formation and growth in ovaries of both the *Ctnnb1;C-Cre* and *Ctnnb1;A-Cre* mouse strains. These results were totally unexpected based on the completely opposite effects of expressing $KRAS^{G12D}$ alone (Fan HY, Shimada M *et al.*, 2008) compared to disrupting *Pten* alone (Fan HY, Liu Z *et al.*, 2008) in granulosa cells. Whereas premature expression of $KRAS^{G12D}$ in granulosa cells of small follicles causes granulosa cell cycle arrest and elevated levels of PTEN in these cells (Fan HY, Shimada M *et al.*, 2008), loss of *Pten* enhances granulosa cell proliferation, leading infrequently to GCTs (Fan HY, Liu Z *et al.*, 2008; Lague MN, Paquet M *et al.*,

2008). Therefore, we predicted that expression of $KRAS^{G12D}$ would block or reduce granulosa cell proliferation and the appearance of precancerous lesions observed in the in the *Ctnnb1;A-Cre* and *Ctnnb1;C-Cre* mutant mice. However, contrary to this hypothesis, the *Ctnnb1;Kras;A-Cre* and *Ctnnb1;Kras;C-Cre* mutant mice develop precancerous lesions similar to those observed in the *Ctnnb1;A-Cre* and *Ctnnb1;C-Cre* mouse strains. Furthermore, GCTs develop in these mice with 100% penetrance. Although the growth rate of the GCTs in the *Ctnnb1;Kras;A-Cre* and *Ctnnb1;Kras;C-Cre* mutant mice is slower than in the *Ctnnb1; Pten;C-Cre* and *Ctnnb1;Pten;A-Cre* mice, the phenotypic outcomes are ultimately similar. Collectively, these results indicate that the potent mechanisms by which $KRAS^{G12D}$ alone acts to block or terminate granulosa cell proliferation in small antral follicles is reversed and/or superceded by events initiated and driven by dominant stable CTNNB1. Moreover, because ovaries of the *Ctnnb1;Pten;A-Cre* mice expressed reduced levels of KRAS and ERK1/2 phosphorylation compared to the *Ctnnb1;C-Cre* and *Ctnnb1;Kras;C-Cre* mice, it appears that the presence of KRAS and ERK1/2 reduces the rate of tumor growth but cannot completely block proliferation as observed when $KRAS^{G12D}$ is expressed alone in the *Kras;C-Cre* mice (Fan HY, Shimada M *et al.*, 2008).

Equally impressive, and supporting the phenotypic outcome of the different *Ctnnb1* mutant strains, was the high degree of similarity among the gene expression profiles of the precancerous lesion- and tumor- bearing ovaries in the *Ctnnb1;Kras;C-Cre* and *Ctnnb1;Kras;A-Cre* and *Ctnnb1;Pten;C-Cre* and *Ctnnb1;Pten;A-Cre* mutant female mice. Many of the genes up-regulated by mutant CTNNB1 alone in the precancerous ovaries were expressed at even higher levels in ovaries of the *Ctnnb1;Kras;C-Cre* and *Ctnnb1;Kras*;A-Cre and *Ctnnb1;Pten;C-Cre* and *Ctnnb1;Pten;A-Cre* mutant mice. Notable among these were negative regulators of the WNT/CTNNB1 pathway observed in our previous studies, including *Wif1, Axin2* and *Ndk1* (Boerboom D, White LD *et al.*, 2006; Fan HY, O'Connor A *et al.*, 2010). Additional CTNNB1 target genes, identified in the more extensive microarray analyses presented in this study, include the positive regulators, such as *Wnt16*, as well as the negative regulators such as *Apcdd1, Enpp2, Etv1, Peg3, Nkd2, Notum* and *Tbx3* of the canonical WNT pathway.

APCDD1 (adenomatosis polyposis coli down-regulated 1) is a membrane-bound glycoprotein interacts with WNT3 and LRP5 to inhibit WNT signaling (Shimomura Y, Agalliu D *et al.*, 2010; Takahashi M, Fujita M *et al.*, 2002). In colorectal cancer cells, up-regulation of the *Apcdd1* gene promotes cell proliferation (Takahashi M, Fujita M *et al.*, 2002). Based on the dramatic induction of *Apcdd1* expression in GCTs of the ovary and testis, it may be a key driver of CTNNB1 in granulosa cell tumor formation and growth. *Enpp2* encodes ectonucleotide pyrophosphatase/phosphodiesterase family member 2 (autotaxin) that is important for lipid signaling, proliferation and cell migration (Tania M, Khan MdA *et al.*, 2010). The Ets variant transcription factor *Etv1* impacts gastrointestinal tumor formation where it is regulated by a KITL/MAPK1/3 pathway (Chi P, Chen Y *et al.*, 2010). *Etv5* is expressed in granulosa and Sertoli cells and regulates *Ret* expression and signaling in the testis (Eo J, Han K *et al.*, 2008; Tyagi G, Carnes K *et al.*, 2009).

Because *Etv5* is a CTNNB1 target gene that increases expression of *Ret*, the increase in *Etv5* likely leads to enhanced activation of this pathway and tumor growth as in other tissues

(Tyagi G, Carnes K *et al.*, 2009). *Cnr1* and *Tbx3* are CTNNB1 target genes known to impact proliferation and pluripotency and thus may also contribute to the GCT formation (Trazzi S, Steger M *et al.*, 2010; Wagner RT, Xu X *et al.*, 2010). *Peg3* is a maternally imprinted gene that has the potential to inhibit apoptosis (Broad KD, Curley JP *et al.*, 2009). CD83 and NFAT5 are normally related to immune-cell functions (Breloer M Fleischer B, 2008; Drews-Elger K, Ortells MC *et al.*, 2009). Their roles in GCTs remain unknown.

The data generated using the two different mouse strains expressing Cre recombinase document unequivocally that CTNNB1 can alter granulosa cell fate decisions and differentiation at early (*Amhr2Cre*) as well as at later (*Cyp19Cre*) stages of follicle development by blocking the expression of many granulosa cell specific marker genes, such as *Cyp19, Nr5a2, Lhcgr* and *Fshr* (Figures 5, 6, 8) (Boerboom D, White LD *et al.*, 2006; Fan HY, O'Connor A *et al.*, 2010). However, the mutant granulosa cells continue to express markers characteristic of granulosa cells at early stages of gonadal development and follicle formation, such as *Wnt4* and *Foxl2*. These results suggest that the CTNNB1 expressing granulosa cells are locked into a specific, primordial stage of differentiation. That these CTNNB1 expressing granulosa cells are highly susceptible to transformation by either $Kras^{G12D}$ or loss of *Pten*, indicates that each potently and rapidly enhances the effects of CTNNB1 in these cells. These results provide strong evidence that if CTNNB1 or other genetic lesions that directly up-regulate the canonical WNT pathway are mutated or deleted, CTNNB1 is likely to be the strong driver irrespective of what other mutations occur. Moreover, many of the genes that were expressed at elevated levels in the GCTs of the ovary and/or testis are known to be targets of CTNNB1 in other cancer cell types and impact cancer cell growth and invasiveness (Chi P, Chen Y *et al.*, 2010; Harper K, Arsenault D *et al.*, 2010; Heinrich MC Corless CL, 2010; Jane-Valbuena J, Widlund HR *et al.*, 2010; Jiang X, Yu Y *et al.*, 2010; Shimomura Y, Agalliu D *et al.*, 2010; Takahashi M, Fujita M *et al.*, 2002; Tania M, Khan MdA *et al.*, 2010; Wagner RT, Xu X *et al.*, 2010).

The molecular mechanisms by which expression of $Kras^{G12D}$ and loss of *Pten* exert such similar effects on the genetic program initiated by stable CTNNB1 remain to be determined. However, increased phosphorylation of CTNNB1 by activated AKT and the loss of FOXO1 may both contribute to enhanced transcriptional activity of CTNNB1 (Boyer A, Goff AK *et al.*, 2009). Furthermore, AKT phosphorylation was increased in both mutant models and expression of specific components of the PI3K and RAS signaling pathways were increased to a similar extent in tumor of the *Ctnnb1;Kras* and *Ctnnb1;Pten* mutant mice indicating that these two pathways converge in the presence of active CTNNB1. This convergence may involve the up-regulation of the proto-oncogenes *Ret* and *Met* that occurred in the double mutant mice and may be mediated by phosphorylation and increased expression of *Etv5* and/or *Etv1*.

An important issue raised by our findings is their potential relatedness to the pathogenesis of adult and juvenile forms of GCTs in women. It has recently been shown that the vast majority of adult-form GCTs in women bear a common mutation (C134W) in the *FOXL2* gene, suggesting a pivotal role for FOXL2 in one or more phases of disease development (Jamieson S, Butzow R *et al.*, 2010; Shah SP, Kobel M *et al.*, 2009). However, it remains to be determined if and how the disease processes initiated by the manipulation of the WNT/

CTNNB1, PI3K/AKT and/or KRAS pathways in our models relates to the mutant FOXL2-driven human disease. Although our data seem to indicate that *Foxl2* mRNA levels are not meaningfully altered in the various transgenic models, FOXL2 activity may be modulated by other means, such as post-translational modifications or cofactor binding. The biological activities of mutant FOXL2 are as yet poorly understood, and only a handful of studies have begun to elucidate the mechanisms by which it may contribute to tumorigenesis (Fleming NI, Knower KC *et al.*, 2010; Kim JH, Yoon S *et al.*, 2011; Lee K, Pisarska MD *et al.*, 2005). It is plausible that mutant FOXL2 initiates cellular processes resulting in the hyperactivation of the WNT/CTNNB1, PI3K/AKT and/or KRAS pathways that may enhance tumor development. For example, FOXL2 and WNT4 are critical for granulosa cell specification, both regulate aromatase and both promote follicle development (Boyer A, Lapointe E *et al.*, 2010; Fan HY, O'Connor A *et al.*, 2010; Fleming NI, Knower KC *et al.*, 2010; Garcia-Ortiz JE, Pelosi E *et al.*, 2009; Moumne L, Batista F *et al.*, 2008; Uda M, Ottolenghi C *et al.*, 2004). If this also occurs in adult GCTs, the mutant cells may be more responsive to WNT/CTNNB1 activation. In addition, KGN cells that are derived presumably from an adult GCT express the FOXL2 C134W mutant and exhibit constitutive activation of ERK1/2 (Steinmetz R, Wagoner HA *et al.*, 2004). Chemical or siRNA disruption of ERK1/2 blocked proliferation indicating that activation (not a mutation) of a RAS-ERK1/2 cascade is critical for the proliferative potential of these cells. Because RAS can activate the PI3K pathway, this pathway may also contribute to the FOXL2 mutant phenotype. Hence it is not yet clear if mutant FOXL2 is sufficient or necessary to drive GCT formation. Another possibility is that the molecular mechanisms involved in tumor development in our mouse models are partially or completely unrelated to FOXL2. In this regard, the juvenile form of GCT in humans is characterized by an earlier (often prepubertal) onset, is not associated with *FOXL2* mutations (Jamieson S, Butzow R *et al.*, 2010; Shah SP, Kobel M *et al.*, 2009) and the molecular mechanisms controlling this GCT form are ill-defined (Jamieson S, Butzow R *et al.*, 2010). It therefore remains to be determined if the mechanisms involved in juvenile GCT development are more closely related to those that are activated in the mouse GCT models described herein, and therefore that the latter could be thought of as being more analogous to the juvenile rather than the adult human GCT disease. Clearly, many more studies are needed to clarify the drivers and helpers involved at various phases of this complex disease in the ovary.

Based on the gene profiling results in the GCTs of the ovary and the observations that cell morphology and tumor architecture are similar in GCTs of the ovary and testis (Boyer A, Paquet M *et al.*, 2009), we anticipated similar patterns of gene expression in the GCTs of the testis and ovary. However, there are distinct as well as similar gene expression patterns between the GCTs in ovary and testis. Because the gene expression profiles of tumors in the *Ctnnb1;Kras;A-Cre* male mice are more similar to those of the ovarian GCTs than are the profiles in the *Ctnnb1;Pten;A-Cre* strain, tumors in the *Ctnnb1;Kras;A-Cre* male mice may appear to be more differentiated towards the granulosa cell phenotype than the tumors of *Ctnnb1;Pten;A-Cre* male mice. The higher levels of *Wnt4* and *Foxl2* mRNA expression in the *Ctnnb1;Kras;A-Cre* testes are consistent with the idea that these cells have assumed a primordial granulosa cell-like genetic program. Their slower growth rate likely relates to the effects of $KRAS^{G12D}$ that impair granulosa cells proliferation in the ovary. Because *Wnt4*

and *Foxl2* but not *Wnt16* are induced in the GCTs of the testis, these factors may serve as critical positive regulators, perhaps with some functions redundant of WNT16 in this context.

Explanations for the major differences between the *Ctnnb1;Pten;A-Cre* and *Ctnnb1;Kras;A-Cre* gene expression profiles in the GCTs of the testis are not completely obvious and are somewhat surprising given the dramatic similarities in the GCTs in the ovaries of mice with these genotypes. However, the differences may relate to when mutant CTNNB1 is first expressed as well as the time of onset and speed of tumor development. For example, tumors in the 5 week *Ctnnb1;Pten;A-Cre* model are approximately the same size as the tumors in the 5 month old *Ctnnb1;Kras;A-Cre* mice. Although these times were selected to compare early growth stages of tumor development across genotypes, selection criteria based on size may overlook critical molecular events being driven by the loss of *Pten* versus the expression of KRAS^{G12D} at specific stages of cell differentiation. Although cell morphology appears similar in both models, cell morphology is not always an indicator of molecular changes. The lack of more dramatic changes in gene expression in the male GCTs may also reflect the presence of more testicular tissue associated with the tumors than in the ovaries. This may “dilute out” some of the genes that are prominently expressed in the tumor cells.

In summary, our results show that stable expression of CTNNB1 in granulosa or Sertoli cells alters their genetic program and predisposes them to oncogenic transformation by either expression of mutant KRAS^{G12D} or loss of PTEN. Because KRAS^{G12D} alone is a potent suppressor of granulosa cell proliferation, these oncogenic effects of KRAS ^{G12D} were unexpected. Moreover, the striking similarities in the gene expression patterns in the GCTs of the ovary and testis when either mutant KRAS^{G12D} is expressed or *Pten* is lost in the mutant CTNNB1 cells provide strong evidence that CTNNB1 is the driver in these contexts and that KRAS^{G12D} and *Pten* loss promote the program set in motion by the CTNNB1. Although the mutations analyzed herein have not yet been linked to adult GCTs in humans, they may be related to juvenile GCTs or to tumors in other tissues where CTNNB1 is mutated. In this regard, these results may have clinical relevance and help explain why tumor growth is so rapid and pervasive in tissues where mutations in *Ctnnb1* as well as alterations in the RAS or PI3K pathways are common.

Materials and Methods

Animals

Immature C57BL/6 mice were obtained from Harlan, Inc. (Indianapolis, IN). Animals were housed under a 14:10h, light:dark schedule and were treated in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Mice expressing *Ctnnb1*$^{tm1Mmt/}$ *Ctnnb1*^{tm1Mmt} (Boerboom D, Paquet M *et al.*, 2005), *Kras*$^{tm4Tyj/}$ *Kras*^{tm4Tyj} (*L-S-L-Kras*^{G12D}) (Tuveson DA, Shaw AT *et al.*, 2004),*Pten*$^{tm1Hwu/}$ *Pten*^{tm1Hwu} (*Pten*$^{fl/fl}$) (Li G, Robinson GW *et al.*, 2002),were used to generate mice harboring granulosa cells specific mutations of each gene alone or in combination using the two cell-specific Cre recombinase strains of mice *Amhr2*$^{tm3(cre)Bhr/+}$) *Amhr2-Cre* (Jamin SP, Arango NA *et al.*, 2002) and Tg(CYP19A1-Cre)1jri (*Cyp19-Cre*) (Fan HY, Shimada M *et al.*, 2008). All the mutant mouse strains are in the C57BL/6 background.

Histology, TUNEL assay, immunohistochemistry and BrdU uptake

Ovaries were fixed in 4% paraformaldehyde, embedded in Optional Cutting Temperature compound (Sakura Finetek USA Inc.) and stored at −70°C before the preparation of 7 μm sections. Serial sections were stained with hemotoxylin and eosin according to established procedures (Fan HY, O'Connor A *et al.*, 2010). The TUNEL assays were done using the ApopTagPlus apoptosis detection kit (Chemicon International. Temecula, CA) as reported previously (Fan HY, Liu Z *et al.*, 2008). Sections were also probed with primary antibodies to specific proteins (FOXO1, BrdU and phosphohistone H3 from Cell Signaling Technology, Danvers, MA); and (BrdU from Sigma Chemical Co, St. Louis, MO) as indicated in the text and secondary Alexa Fluor 594- or 488-conjugated goat anti-rabbit IgG antibodies (Molecular Probes) as previously described (Fan HY, Liu Z *et al.*, 2009b). Slides were mounted using VectaShield with DAPI (Vector Laboratories). Digital images were captured using a Zeiss Axioplan 2 microscope with 5–63× objectives. For all the experiments, exposure time was kept the same for control and mutant samples. Proliferation was analyzed by BrdU incorporation into cells of mice injected with 50mg/kg BrdU in saline 2 hr prior to sacrifice. Ovaries were embedded as above, sections and immno-stained for BrdU (Sigma Chemical Company, St. Louis, MO).

Western blot analyses

Cell extracts containing 30μg protein were resolved by SDS-PAGE and transferred to PVDF membranes (Millipore Corp., Bedford, MA) and analyzed as previously (Fan HY, Liu Z *et al.*, 2009b) using primary antibodies to CTNNB1 (from Santa Cruz Biotechnology, Inc.) and PTEN, phospho90RSK1/2/3, phospho-ERK1/2, phosphoAKT, phosphoGSK3β, FOXL2 (from Cell Signaling Technology) and ACTIN (from Cytoskeleton, Inc, Denver, CO) at 1:1000 dilutions or as indicated in the Figure legend.

RNA isolation, Microarray analyses and quantitative (Q)PCR

Total RNA was isolated using the RNeasy Mini kit (Qiagen Sciences, Germantown, MD). RNA quality was assessed and then riboprobes were generated from WT and mutant RNA and hybridized to Mouse 430.2 microarray chips (Affymetrix, Santa Clara, CA) in the Microarray Core Facility of the Baylor College of Medicine as previously described (Hernandez-Gonzalez I, Gonzalez-Robayna IJ *et al.*, 2006). Microarray data were analyzed as previously reported using the Robust Multi-array Averaging function (Irizarry RA, Hobbs B *et al.*, 2003) from the Affy package (v1.5.8) through the BioConductor software (http://www.bioconductor.org/ (Gentleman RC, Carey VJ *et al.*, 2004)). The microarray data have been deposited to GEO; the accession number is GSE27656. http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=blczregkwwekmrw&acc=GSE27656

Reverse transcription was done using the SuperScript One-Step RT-PCR system with Platinum Taq kit (Invitrogen, Carlsbad, CA). The QPCR was performed using the Rotor-Gene 3000 thermocycler (Corbett Research, Sydney, Australia). Relative levels of mRNAs were calculated using Rotor-Gene 6.0 software and normalized to the levels of endogenous beta-actin in the same samples.

Statistical analyses

The data are represented as means ± SEM. QPCR data is expressed at the ratio of WT (n=1) to mutant. Data were analyzed by using GraphPad Prism Programs (ANOVA or t-test; GraphPad Prism, San Diego, CA) and Dunnett's post-hoc test after ANOVA to compare all genotypes to the control. Values were considered significantly different if $P \leq 0.05$ or $P \leq 0.01$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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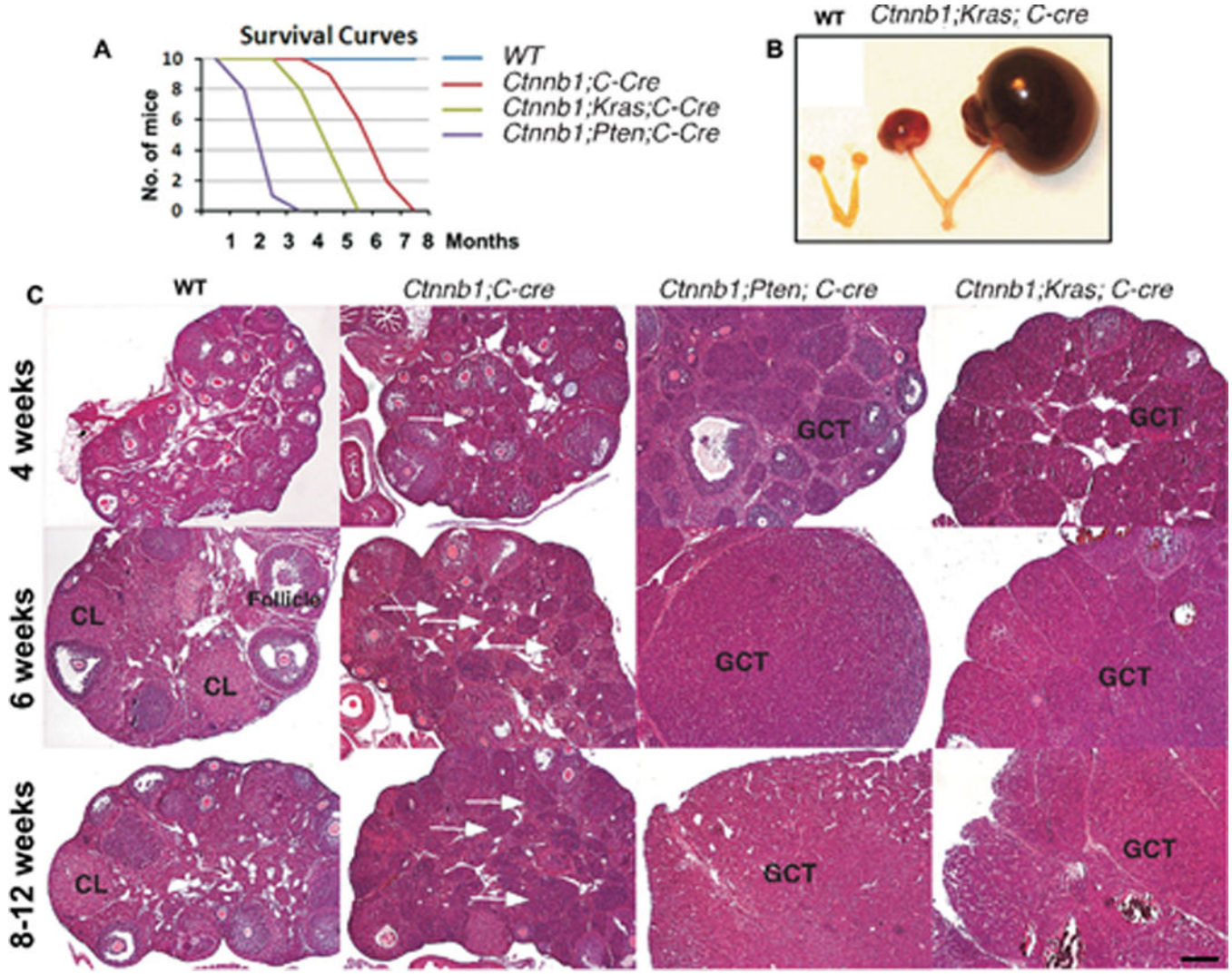

Figure 1.

Ovarian granulosa cell tumor formation is enhanced when mutant active CTNNB1 is co-expressed with mutant $KRAS^{G12D}$ or in the absence of PTEN. *Ctnnb1;C-Cre* mice as well as *Ctnnb1;Kras;C-Cre* and *Ctnnb1;Pten C-Cre* mice were generated to determine the effects of stable active $KRAS^{G12D}$ and the loss of *Pten* in the mice expressing stable active CTNNB1 selectively in granulosa cells of growing follicles. **A:** Survival rates for mice of the different genotypes indicates that the *Ctnnb1;Pten;C-Cre* mice succumbed to tumor volume first followed by the *Ctnnb1; Kras;C-Cre* mice and the *Ctnnb1;C-Cre* mice. **B**: Gross morphology of tumor-bearing ovaries present in a *Ctnnb1;Kras;C-Cre* mouse at 3 months compared to a WT mouse at the same age. **C**: Histological sections of ovaries from WT and mutant mice at different ages. The images show normal follicles and corpora lutea (CL) in WT mice, precancerous lesions (white arrows) in the *Ctnnb1;C-Cre* mice and tumors (GCTs) in the *Ctnnb1; Kras;C-Cre* and *Ctnnb1;Pten;C-Cre* mice. Tumors of the *Ctnnb1;Pten;C-Cre* mice grow faster than those expressing $Kras^{G12D}$.

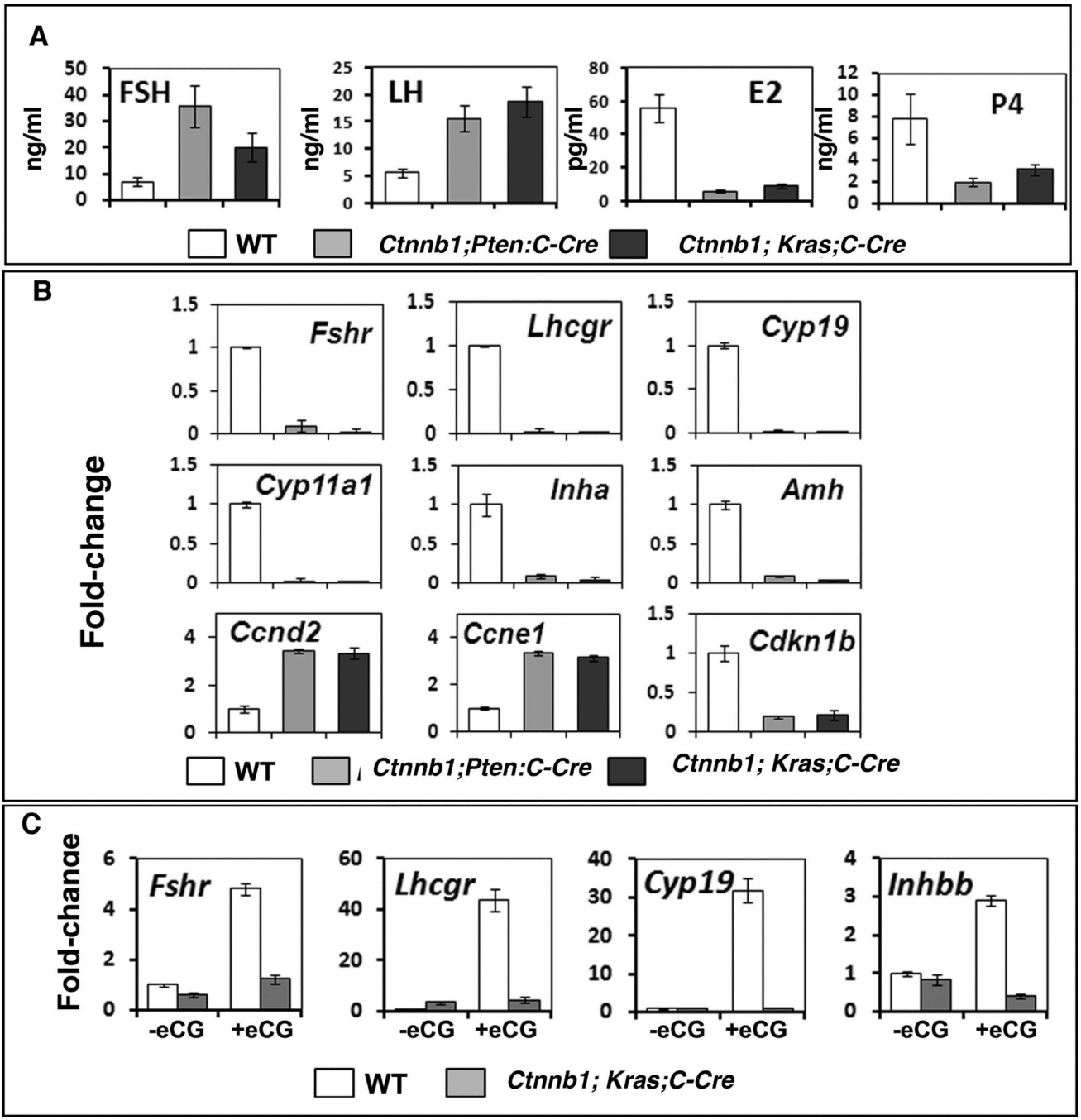

Figure 2.

Granulosa cell differentiation is blocked in the double mutant mice. **A**: Serum levels of FSH and LH are elevated whereas estradiol and progesterone are low in the *Ctnnb1;Pten ;C-Cre* and *Ctnnb1;Kras;C-Cre* mice compared to controls at 6–8 weeks of age. **B**: Granulosa cell specific marker genes are suppressed in ovaries of the *Ctnnb1;Pten ;C-Cre* and *Ctnnb1;Kras;C-Cre* mutant mice that contain GCTs. Genes controlling cell proliferation are increased whereas a cell cycle inhibitor is decreased. **C**: Induction of granulosa cell marker

genes in immataure mice by treatment with eCG (48h) is suppressed in the *Ctnnb1; Kras;C-Cre* mice compared to WT.

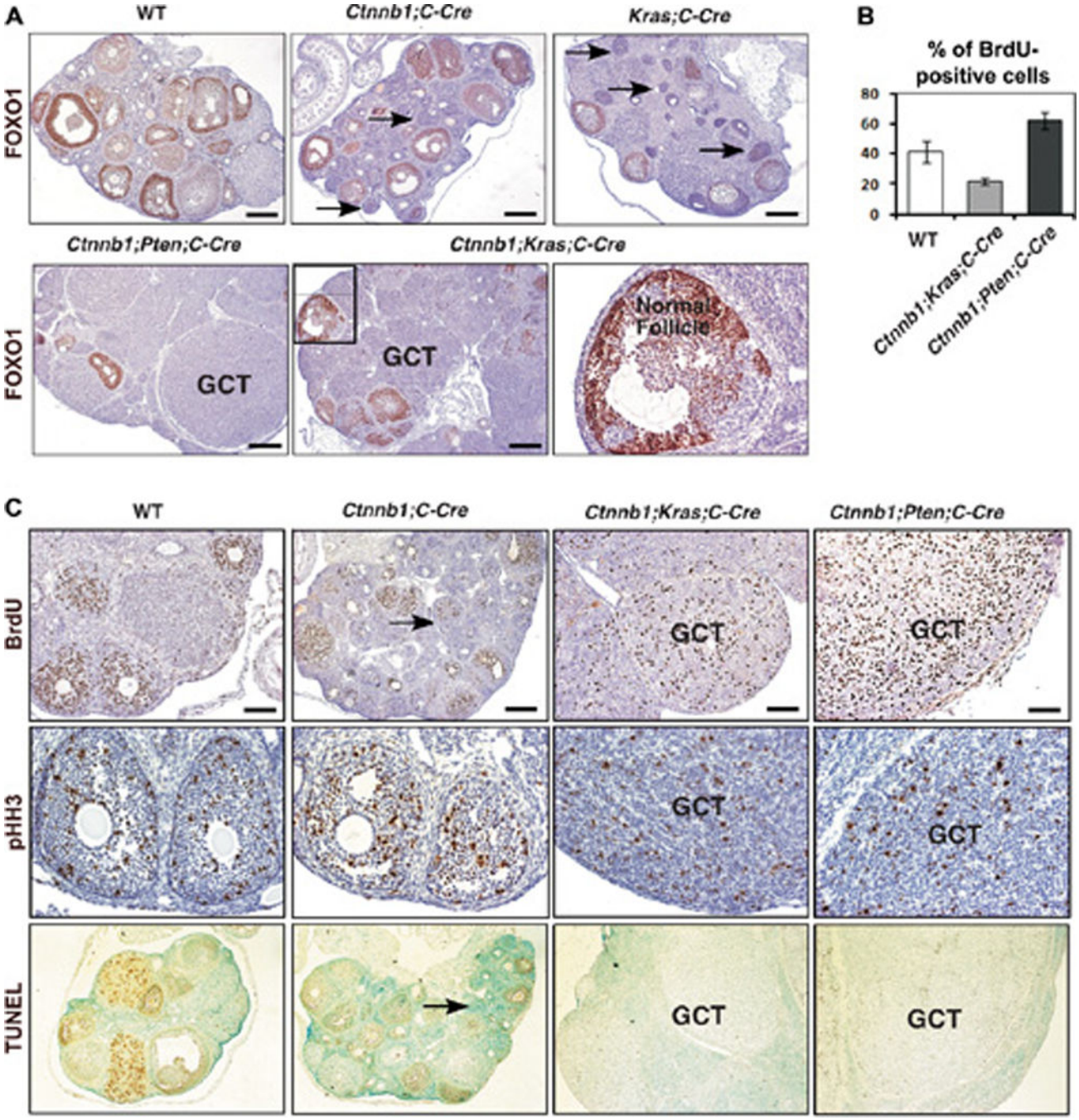

Figure 3.

Proliferation and apoptosis are altered in the GCTs of the mutant mice at 6 weeks of age. **A**: Immunostaining of FOXO1 is high in growing follicles present in the ovaries of WT and mutant mice but is absent in the precancerous lesions (black arrows) of the *Ctnnb1;C-Cre* mice, the abnormal follicles (black arrows) present in ovaries of *Kras;C-Cre* mice and the GCTs of the double mutant mice. **B** and **C**: Granulosa cell proliferation (BrdU uptake and phospho histone H3 staining) is enhanced in the GCTs of the *Ctnnb1;Pten;C-Cre* mice compared to WT and *Ctnnb1;Kras;C-Cre* mice whereas apoptosis is reduced in the GCTs.

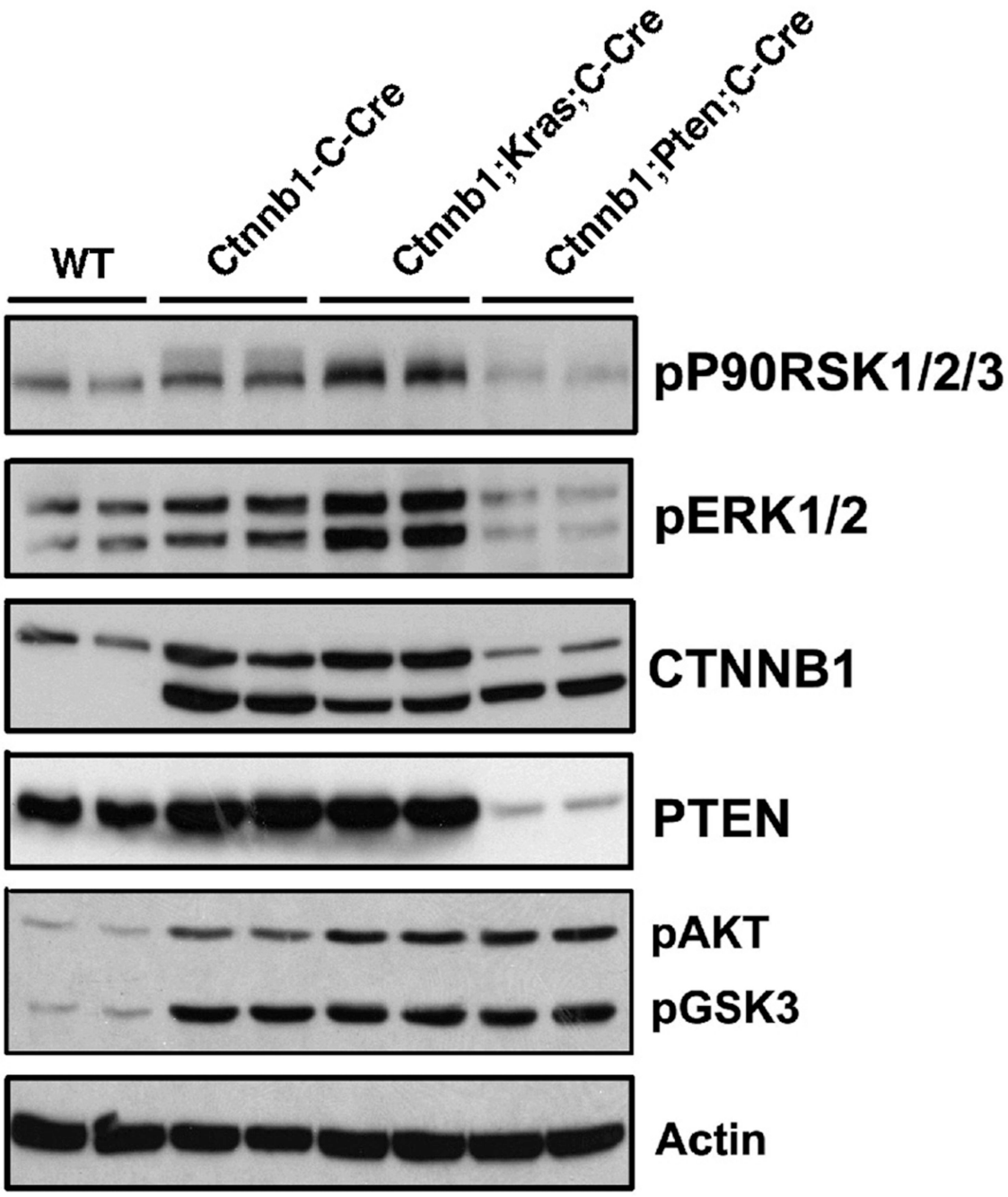

Figure 4.

Expression and activation of signaling pathways in WT and mutant ovaries at 4 weeks of age. Western blot shows that elevated levels of CTNNB1 alone or in the presence of $KRAS^{G12D}$ or with the loss of *Pten* are associated with increased phosphorylation of both AKT and GSK3. Phosphorylation of p90RSK and ERK1/2 are highest in the *Ctnnb1;C-Cre* and *Ctnnb1; Kras;C-Cre* mice.

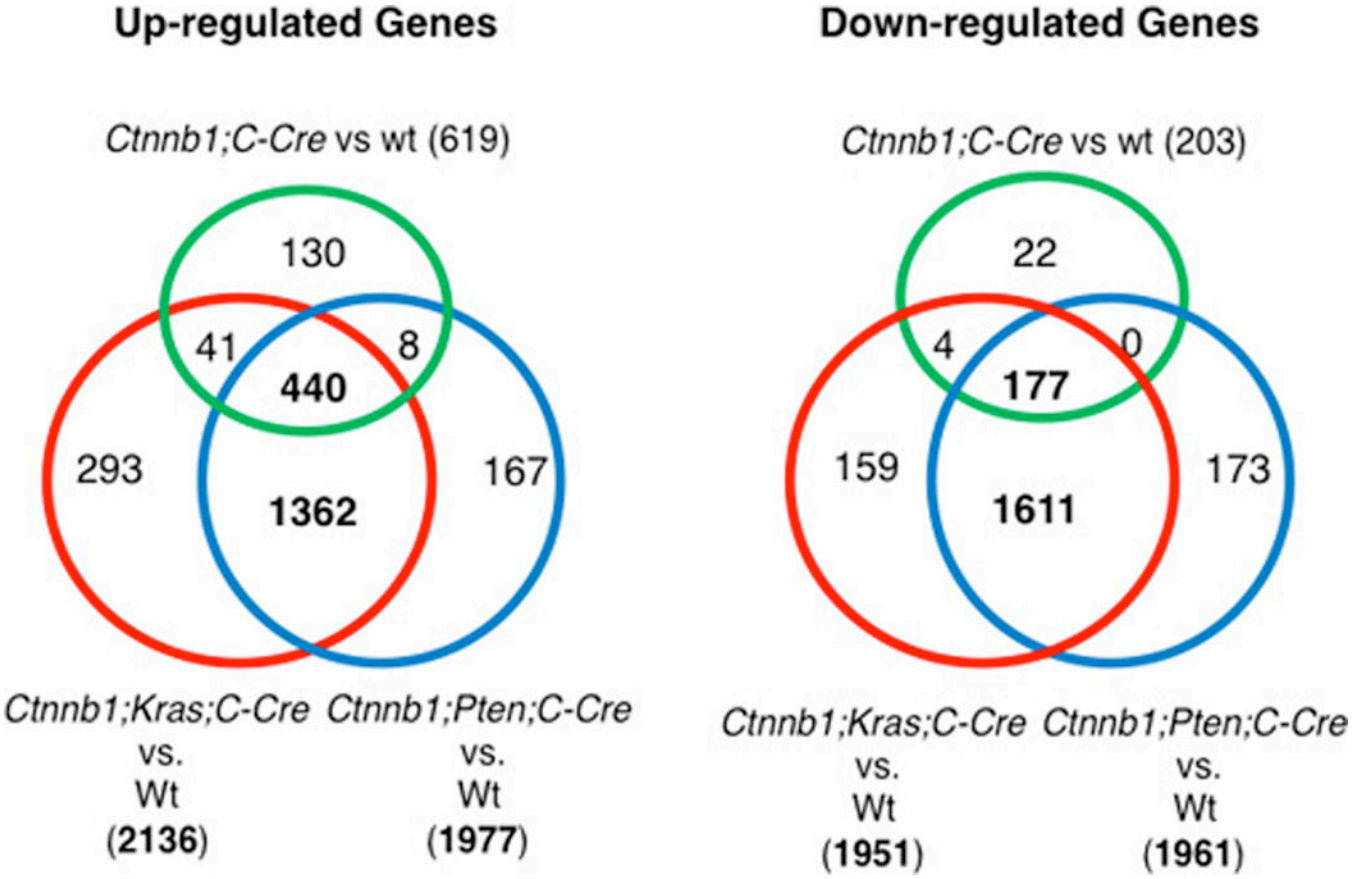

Figure 5.

Specific and overlapping gene expression profiles characterize the mutant ovaries. Based on the microarray data, ovaries of *Ctnnb1;C-Cre* mice exhibited up-regulation of (130) and down-regulation of (22) a limited number of genes, respectively. In *Ctnnb1;Kras;C-Cre* and *Ctnnb1;Pten;C-Cre* mouse ovaries, there are overlapping gene expression profiles of genes up-regulated (440) and down-regulated (177) in the *Ctnnb1;C-Cre* mouse ovaries. In addition, there is a large number of genes are up-regulated (1362) or down-regulated in ovaries of both the *Ctnnb1;Kras;C-Cre* and *Ctnnb1;Pten;C-Cre* mutant mice.

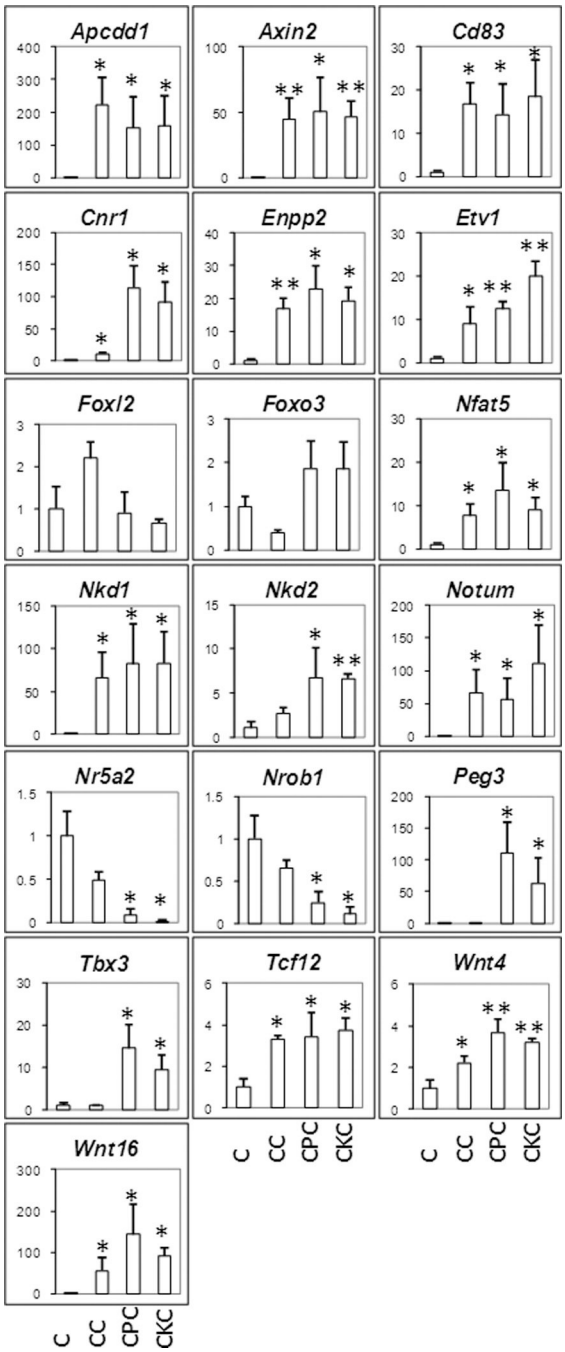

Figure 6.

Gene expression patterns in mutant ovaries were verified by real-time RT-PCR. C, control, KC, *Kras;C-Cre*, PC, *Pten;C-Cre*, CKC, *Ctnnb1;Kras;C-Cre*, CPC, *Ctnnb1; Pten;C-Cre*. Each graph show averages of n=4 (columns) ± SEM (errors bars) for each genotype. Statistically significant differences with control (A) are indicated with an asterisk (*)($P < 0.05$) or a double asterisk (**)($P<0.01$).

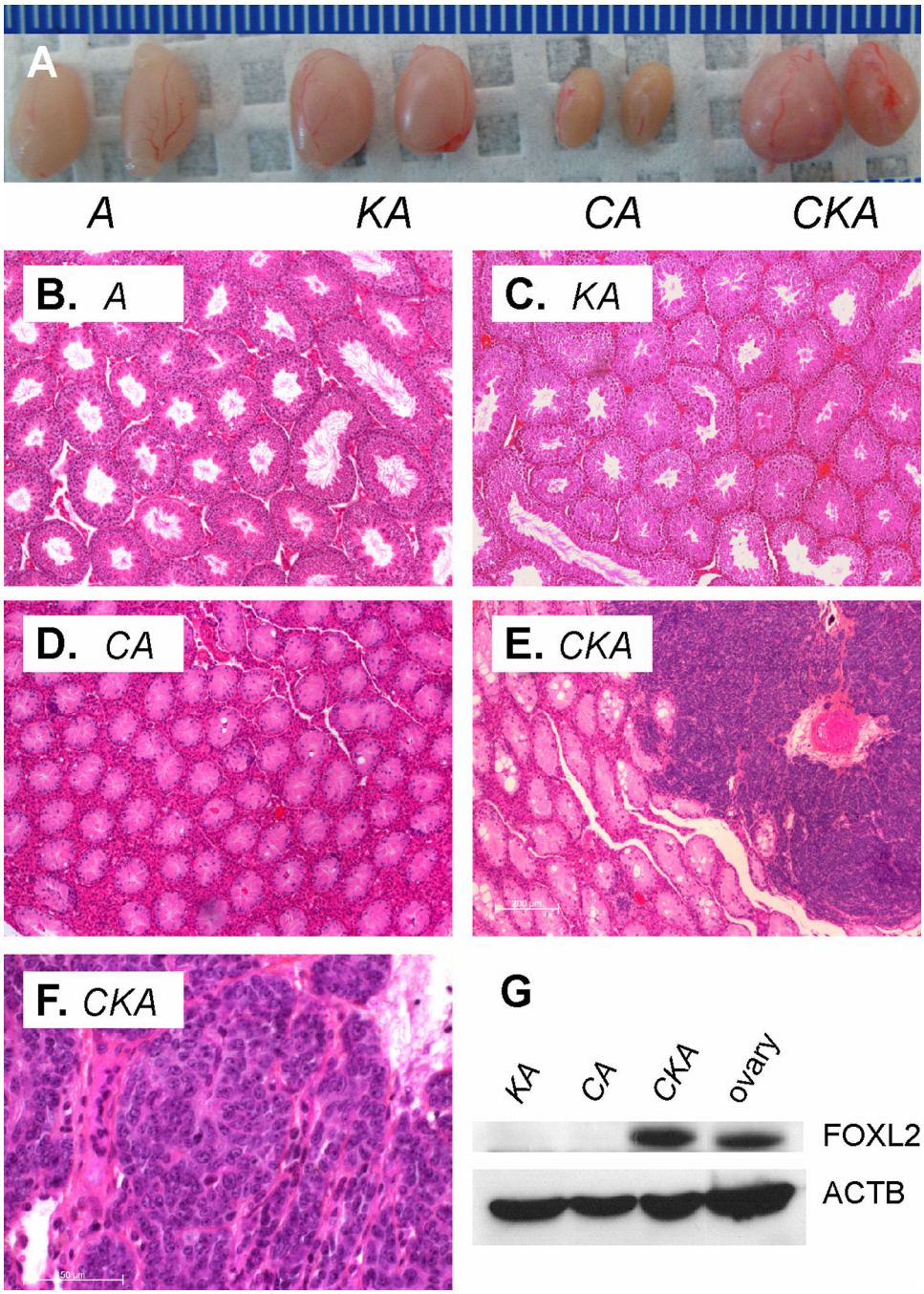

Figure 7.

Ctnnb1;Kras;A-Cre mice develop GCTs of the testis. Gross (A) and histological (B–F) images of testes of the indicated genotypes (A, *A-Cre* (control), KA, *Kras;A-Cre*, CA, *Ctnnb1;A-Cre*, CKA, *Ctnnb1;Kras;A-Cre*). Scale bar for panels B–E is located in panel E. G, Western blotting analysis of FOXL2 expression in testes of the indicated genotypes (as above), along with a sample of an ovary from a wild-type mouse. ACTB was used as a loading control.

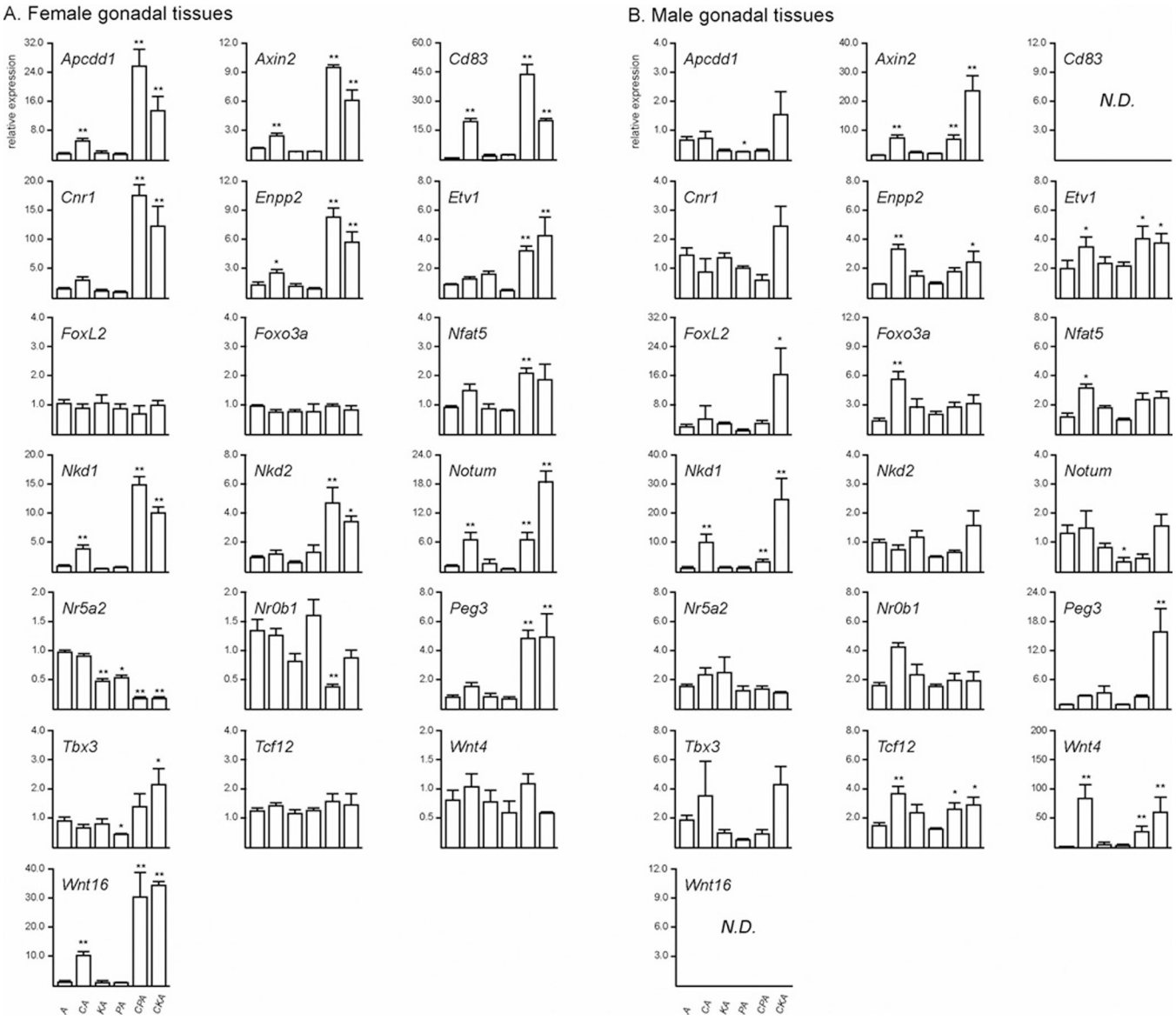

Figure 8.

Gene expression profiles in GCTs of the ovary and testis.

C, control, KA, *Kras;A-Cre*, PA, *Pten;A-Cre*, CKA, *Ctnnb1;Kras;A-Cre*, CPA, *Ctnnb1;Pten;A-Cre*. Each graph show averages of n=4 (columns) ± SEM (errors bars) for each genotype. Statistically significant differences with control (A) are indicated with an asterisk (*)($P < 0.05$) or a double asterisk (**)($P<0.01$).

Table 1

Gene expression profiles in *Ctnnb1*, *Kras/Pten*, *Ctnnb1/Kras* and *Ctnnb1/Pten* mutant mice

RNA was extracted from whole ovaries of control (WT) mice, *Ctnnb1-Cyp19-cre*, *Kras;Pten-Cyp19-cre*, *Ctnnb1;Kras-Cyp19-cre* and *Ctnnb1;Pten-Cyp19-cre* mice at 6 weeks of age. The RNA samples (2 /genotype) were analyzed in duplicate using the Affymetrix Mouse 430.2 array chip. All mutant mRNA values were expressed relative to that detected in WT samples.

Probe Set ID	Gene Title	Gene Symbol	*Ctnnb 1*/wt	*Kras; Pten*/ wt	*Ctnnb 1;Kras* /wt	*Ctnnb1; Pten*/ wt
Top10 Down Regulated						
1418979_at	aldo-keto reductase family 1, member C14	Akr1c14	−1.6	−3.9	−60	−139.2
1416434_at	Bcl2-like 10	Bcl2l10	−3.3	−78.4	−80	−80
1460258_at	leukocyte cell derived chemotaxin 1	Lect1	−2.3	−32.2	−139.1	−139.1
1417411_at	nucleosome assembly protein 1-like 5	Nap1l5	−1.6	−53.1	−63.3	−132.3
1429409_at	NLR family, pyrin domain containing 14	Nlrp14	−4	−93.6	−93.6	−93.6
1420410_at	nuclear receptor subfamily 5, group A, member 2	Nr5a2	−4.5	−51.5	−88.8	−122.6
1419663_at	osteoglycin	Ogn	1	−1.8	−86.1	−155.6
1427976_at	oogenesin 1	Oog1	−4.1	−127.8	−148.2	−148.2
1436279_at	Solute carrier family 26, member 7	Slc26a7	−1.7	−3.1	−111.7	−111.7
1454622_at	solute carrier family 38, member 5	Slc38a5	−3.5	−116.2	−146.4	−146.4
Top10 Up Regulated						
1450770_at	RIKEN cDNA 3632451O06 gene	3632451O06Rik	27.2	−2.1	74.9	80.9
1418382_at	adenomatosis polyposis coli down-regulated 1	Apcdd1	49.8	−1.6	74.9	75.6
1423286_at	cerebellin 1 precursor protein	Cbln1	2.5	1	108.2	120.1
1434172_at	cannabinoid receptor 1 (brain)	Cnr1	15.4	1	79.2	73.5
1448136_at	ectonucleotide pyrophosphatase/phosphodiesterase 2	Enpp2	29.5	9.2	70.1	69
1455645_at	myosin binding protein C, slow-type	Mybpc1	34.7	1.2	161.8	140.2
1451857_a_at	notum pectinacetylesterase homolog (Drosophila)	Notum	61.3	1.7	80.2	75.1
1439500_at	secernin 1	Scrn1	21	6.8	89.8	91
1460244_at	ureidopropionase, beta	Upb1	4.9	1	106.5	70.4
1425425_a_at	Wnt inhibitory factor 1	Wif1	57.6	1	73.8	89.2
WNT Pathway						

Probe Set ID	Gene Title	Gene Symbol	*Ctnnb 1*/wt	*Kras; Pten*/ wt	*Ctnnb 1;Kras* /wt	*Ctnnb1; Pten*/ wt
1436845_at	axin2	Axin2	10.7	1.4	16.5	17.1
1448698_at	cyclin D1	Ccnd1	5.4	6.1	7	7.1
1434745_at	cyclin D2	Ccnd2	1.1	−1.1	2.2	2.1
1416111_at	CD83 antigen	Cd83	17.9	3.3	18.6	15.6
1437351_at	CXXC finger 4	Cxxc4	1.4	−4.3	−7.9	−7.3
1417937_at	dapper homolog 1, antagonist of beta-catenin (xenopus)	Dact1	4.4	−1.8	7.4	7.3
1420512_at	dickkopf homolog 2 (Xenopus laevis)	Dkk2	2.3	5.4	5.3	4.8
1448669_at	dickkopf homolog 3 (Xenopus laevis)	Dkk3	3	2.2	6.2	5.7
1425447_at	dickkopf homolog 4 (Xenopus laevis)	Dkk4	15.1	1	1	1
1422607_at	ets variant gene 1	Etv1	2.5	1.5	6.4	6.8
1428142_at	ets variant gene 5	Etv5	23.2	4.5	16.5	18.1
1437284_at	frizzled homolog 1 (Drosophila)	Fzd1	4.1	8.8	5.5	5.9
1419301_at	frizzled homolog 4 (Drosophila)	Fzd4	−1.5	3.7	−2.3	−2.2
1417301_at	frizzled homolog 6 (Drosophila)	Fzd6	1.7	1.5	−2.2	−2.8
1423348_at	frizzled homolog 8 (Drosophila)	Fzd8	−1.2	−1.1	−4.8	−5
1454734_at	lymphoid enhancer binding factor 1	Lef1	4.3	1.1	14.4	16.1
1448342_at	mitogen-activated protein kinase 10	Mapk10	−1.9	−3.2	−5.5	−5
1438999_a_at	nuclear factor of activated T-cells 5	Nfat5	8.3	1.4	9.9	9.1
1439205_at	nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 2	Nfatc2	11.6	4.2	21.9	26
1429506_at	naked cuticle 1 homolog (Drosophila)	Nkd1	23.3	1	32.5	30.6
1419466_at	naked cuticle 2 homolog (Drosophila)	Nkd2	1.2	1.7	11.1	7.8
1435970_at	nemo like kinase	Nlk	1.9	1.1	3.3	3.4
1451857_a_at	notum pectinacetylesterase homolog (Drosophila)	Notum	61.3	1.7	80.2	75.1
1417355_at	paternally expressed 3	Peg3	5.6	2.9	12.7	15.7
1437393_at	protein kinase C, alpha	Prkca	1.7	2.4	3.5	3.2
1460419_a_at	protein kinase C, beta	Prkcb	4.9	6.7	29.6	31.6
1449319_at	R-spondin homolog (Xenopus laevis)	Rspo1	1.2	14.9	−1.8	−1.3
1455893_at	R-spondin 2 homolog (Xenopus laevis)	Rspo2	−4.4	−24.6	−28.5	−28.5

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Probe Set ID	Gene Title	Gene Symbol	*Ctnnb 1*/wt	*Kras; Pten*/ wt	*Ctnnb 1;Kras* /wt	*Ctnnb1; Pten*/ wt
1455607_at	R-spondin 3 homolog (Xenopus laevis)	Rspo3	20.3	6.9	7	4.5
1428136_at	secreted frizzled-related protein 1	Sfrp1	1	7.2	−8.8	−14.6
1448201_at	secreted frizzled-related protein 2	Sfrp2	3	5.6	1	1
1451031_at	secreted frizzled-related protein 4	Sfrp4	50.8	14.1	1.3	−1.4
1416564_at	SRY-box containing gene 7	Sox7	1.7	2.1	7.3	6.6
1448029_at	T-box 3	Tbx3	11.3	1.1	31.6	31.1
1433471_at	transcription factor 7, T-cell specific	Tcf7	5.7	−2.5	4.7	4.9
1455256_at	TRAF2 and NCK interacting kinase	Tnik	3.7	1	6	6
1425425_a_at	Wnt inhibitory factor 1	Wif1	57.6	1	73.8	89.2
1422941_at	wingless-related MMTV integration site 16	Wnt16	5.9	1	60.2	38.3
1449425_at	wingless-related MMTV integration site 2	Wnt2	3.8	2	4.3	4.2
1450782_at	wingless-related MMTV integration site 4	Wnt4	7.3	4.8	7	6.6
1436791_at	wingless-related MMTV integration site 5A	Wnt5a	3.2	3.4	2.9	3.1
1422602_a_at	wingless-related MMTV integration site 5B	Wnt5b	4	1.7	7.3	8.6
Granulosa Cell Markers						
1448804_at	cytochrome P450, family 11, subfamily a, polypeptide 1	Cyp11a1	4.3	−1.1	−8.1	−65.2
1449920_at	cytochrome P450, family 19, subfamily a, polypeptide 1	Cyp19a1	3.3	−8.5	−4.9	−6.8
1460591_at	estrogen receptor 1 (alpha)	Esr1	1.4	5.5	4.3	4.1
1426103_a_at	estrogen receptor 2 (beta)	Esr2	−3.4	−11.9	−15.5	−15.5
1450810_at	follicle stimulating hormone receptor	Fshr	−2.2	−10.5	−17.1	−14.9
1434458_at	follistatin	Fst	−2.5	−7.5	−5.1	−4.8
1422728_at	inhibin alpha	Inha	−1.1	−4.4	−33.5	−66.5
1422053_at	inhibin beta-A	Inhba	1.2	−3.3	−3.1	−3.3
1426858_at	inhibin beta-B /// similar to Inhbb protein	Inhbb	−1.5	−6.6	−15.3	−20.7
1450192_at	luteinizing hormone/choriogonadotropin receptor	Lhcgr	4.2	2.8	−4.5	−4.5
1417760_at	nuclear receptor subfamily 0, group B, member 1	Nr0b1	−2.9	−2.2	−12.8	−12.8
1420410_at	nuclear receptor subfamily 5, group A, member 2	Nr5a2	−4.5	−51.5	−88.8	−122.6
Oocyte Markers						

Probe Set ID	Gene Title	Gene Symbol	*Ctnnb 1*/wt	*Kras; Pten*/ wt	*Ctnnb 1;Kras* /wt	*Ctnnb1; Pten*/ wt
1448120_at	growth differentiation factor 9	Gdf9	−4.3	−30.3	−38.3	−75.2
1460123_at	G protein-coupled receptor 1	Gpr1	−5.7	−9.8	−11.5	−11.5
1416518_at	H1 histone family, member O, oocyte-specific	H1foo	−3.7	−6.8	−6.8	−6.8
1429409_at	NLR family, pyrin domain containing 14	Nlrp14	−4	−93.6	−93.6	−93.6
1438077_at	NLR family, pyrin domain containing 4A	Nlrp4a	−7.4	−20.8	−20.8	−20.8
1434527_at	NLR family, pyrin domain containing 4B	Nlrp4b	−5.9	−11.3	−11.3	−11.3
1436794_at	NLR family, pyrin domain containing 4F	Nlrp4f	−4.5	−32.2	−32.2	−32.2
1418882_at	NLR family, pyrin domain containing 5	Nlrp5	−3.3	−28	−33.3	−23.5
1425759_at	NOBOX oogenesis homeobox	Nobox	−5.4	−8.7	−7	−6.8
1456305_x_at	similar to OBOX2 /// oocyte specific homeobox 2	Obox2	−6.4	−70.1	−70.1	−70.1
1436741_at	oocyte specific homeobox 5	Obox5	−6.2	−18	−25.7	−26.7
1437732_at	oocyte maturation, alpha	Omt2a	−5	−5	−5	−5
1447499_s_at	oocyte maturation, alpha /// oocyte maturation, beta	Omt2a /// Omt2b	−4.6	−52	−52	−52
1455041_at	oocyte maturation, beta	Omt2b	−5.1	−14.2	−14.2	−14.2
1460471_at	oocyte expressed protein homolog (dog)	Ooep	−3.8	−19.5	−26.5	−26.5
1427976_at	oogenesin 1	Oog1	−4.1	−127.8	−148.2	−148.2
1437153_at	predicted gene 13084 /// oogenesin 2	Oog2	−5.1	−63.8	−63.8	−63.8
1436720_s_at	oogenesin 3	Oog3	−5.7	−37.3	−37.3	−37.3
1456183_at	oogenesin 4	Oog4	−6.5	−33.4	−34.1	−34.1
1418531_at	oocyte secreted protein 1	Oosp1	−5.1	−57.8	−57.8	−57.8
1450306_at	zona pellucida glycoprotein 1	Zp1	−5.3	−13.8	−19	−19
1449016_at	zona pellucida glycoprotein 2	Zp2	−4.2	−61.5	−58	−55.7
1419007_at	zona pellucida glycoprotein 3	Zp3	−5.1	−58.4	−58.4	−58.4
IGF/PI3K/AKT Pathway						
1448698_at	cyclin D1	Ccnd1	5.4	6.1	7	7.1
1423100_at	FBJ osteosarcoma oncogene	Fos	−4.5	1.7	−16	−37.6
1416982_at	forkhead box O1	Foxo1	−2.4	−6.5	−8.1	−8.3
1425458_a_at	growth factor receptor bound protein 10	Grb10	1	1.1	−2.5	−4.9

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Probe Set ID	Gene Title	Gene Symbol	*Ctnnb 1*/wt	*Kras; Pten*/ wt	*Ctnnb 1;Kras* /wt	*Ctnnb1; Pten*/ wt
1422943_a_at	heat shock protein 1	Hspb1	2.8	6.2	5	4.1
1437401_at	insulin-like growth factor 1	Igf1	−1.6	1	−19.7	−23.2
1452982_at	insulin-like growth factor I receptor	Igf1r	−1.6	−4.3	−5.8	−7.5
1443969_at	insulin receptor substrate 2	Irs2	−2	−3.8	−3.6	−2.3
1443798_at	phosphatidylinositol 3-kinase catalytic delta polypeptide	Pik3cd	−7.5	−5.5	−5	−5.5
1456482_at	phosphatidylinositol 3 kinase, regulatory subunit, polypeptide 3 (p55)	Pik3r3	2.3	4.1	3.9	4.2
1460352_s_at	phosphatidylinositol 3 kinase, regulatory subunit, polypeptide 4, p150	Pik3r4	1.5	−1.5	2	2
1437393_at	protein kinase C, alpha	Prkca	1.7	2.4	3.5	3.2
1460419_a_at	protein kinase C, beta	Prkcb	4.9	6.7	29.6	31.6
1435698_at	RPTOR independent companion of MTOR, complex 2	Rictor	2	1	2.5	2.3
1416896_at	ribosomal protein S6 kinase polypeptide 1	Rps6ka1	2.7	1.4	5.4	5.2
1449109_at	suppressor of cytokine signaling 2	Socs2	−1.7	−4.2	−48.8	−67.5
1422458_at	T-cell lymphoma breakpoint 1	Tcl1	−4.1	−56.2	−57.3	−57.3
1418162_at	toll-like receptor 4	Tlr4	1.8	45.1	11.7	9.1
1423047_at	toll interacting protein	Tollip	2	1.5	3.2	2.9
Ras Signaling						
1451159_at	Rho guanine nucleotide exchange factor (GEF) 12	Arhgef12	1.8	2.9	2.3	2.1
1421042_at	rho/rac guanine nucleotide exchange factor (GEF) 2	Arhgef2	1.4	1.4	2.6	2.7
1424250_a_at	Rho guanine nucleotide exchange factor (GEF) 3	Arhgef3	1.9	1	2.1	2.3
1452304_a_at	Rho guanine nucleotide exchange factor (GEF) 5	Arhgef5	1.8	2.6	2.6	2.6
1422824_s_at	epidermal growth factor receptor pathway substrate 8	Eps8	−1.1	2.1	−18.3	−19.1
1438097_at	RAB20, member RAS oncogene family	Rab20	2.9	5.2	3.3	2
1421872_at	RAB24, member RAS oncogene family	Rab24	2.1	1.6	2.8	2.6
1448304_a_at	RAB6, member RAS oncogene family	Rab6	2.2	1.3	2.1	2
1425518_at	Rap guanine nucleotide exchange factor (GEF) 4	Rapgef4	1.6	−6.2	2.2	2.1
1439622_at	Ras association (RalGDS/AF-6) domain family member 4	Rassf4	−2.1	1.1	−4.1	−5.5
1452283_at	Ras association (RalGDS/AF-6) domain family (N-terminal) member 8	Rassf8	2.3	1.9	2.7	3.1
1422562_at	Ras-related associated with diabetes	Rrad	5.2	3.4	3.4	4.3

Oncogene. Author manuscript; available in PMC 2012 September 22.

Probe Set ID	Gene Title	Gene Symbol	*Ctnnb 1*/wt	*Kras; Pten*/ wt	*Ctnnb 1;Kras* /wt	*Ctnnb1; Pten*/ wt
Oncogene						
1423100_at	FBJ osteosarcoma oncogene	Fos	−4.5	1.7	−16	−37.6
1451716_at	v-maf musculoaponeurotic fibrosarcoma oncogene family, protein B (avian)	Mafb	4.4	4.6	3.2	3.1
1434447_at	met proto-oncogene	Met	1.9	7.9	12.6	14.2
1450775_at	Moloney sarcoma oncogene	Mos	−4.8	−15.6	−15.6	−15.6
1434777_at	v-myc myelocytomatosis viral oncogene homolog 1, lung carcinoma derived (avian)	Mycl1	−5.2	−18.6	−22.7	−23.5
1417700_at	RAB38, member of RAS oncogene family	Rab38	−2.8	−2.3	−6.3	−6.3
1436359_at	ret proto-oncogene	Ret	1.3	1	20.7	22.2
1455425_at	tet oncogene 1	Tet1	1.7	−2.3	4.2	4.1

Table 2

Gonadal weights and ages of mice in each of the mutant mice genotypes: *A-Cre* (control), *Ctnnb1;A-Cre* (CA), *Kras;A-Cre* (KA), *Pten A-Cre* (PA), *Ctnnb1;Pten ACre*(CPA) and *Ctnnb1;Kras;A-Cre* (CKA) mutant mice. Note that tumors occurred earlier and grew faster (based on gonad weight) in the *Ctnnb1;Pten A-Cre* (CPA) male and female mice compared to the *Ctnnb1;Kras;A-Cre* (CKA) mutant mice.

	Control	**CA**	**KA**	**PA**	**CPA**	**CKA**
TESTIS	97.8+/−1.3	**27.4+/−3.7***	87.0+/−4.1	108.3+/−4.3	**52.3+/−7.1***	**51.7+/−14.2***
Age:	11weeks	9–14weeks	10–11weeks	9–13weeks	**5weeks**	**4–5.5 months**
OVARY	3.9+/− 0.4	5.0+/−0.4	**10.0+/−0.6***	**6.5+/−0.8***	**18.3+/−2.2***	**23.4+/− 4.7***
Age:	11weeks	9–12weeks	8–14weeks	13weels	**3weeks**	**12–14weeks**