Coexistent ARID1A–PIK3CA mutations promote ovarian clear-cell tumorigenesis through pro-tumorigenic inflammatory cytokine signalling

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Ovarian clear-cell carcinoma (OCCC) is an aggressive form of ovarian cancer with high ARID1A mutation rates. Here we present a mutant mouse model of OCCC. We find that ARID1A inactivation is not sufficient for tumour formation, but requires concurrent activation of the phosphoinositide 3-kinase catalytic subunit, PIK3CA. Remarkably, the mice develop highly penetrant tumours with OCCC-like histopathology, culminating in haemorrhagic ascites and a median survival period of 7.5 weeks. Therapeutic treatment with the pan-PI3K inhibitor, BKM120, prolongs mouse survival by inhibiting the tumour cell growth. Cross-species gene expression comparisons support a role for IL-6 inflammatory cytokine signalling in OCCC pathogenesis. We further show that ARID1A and PIK3CA mutations cooperate to promote tumour growth through sustained IL-6 overproduction. Our findings establish an epistatic relationship between SWI/SNF chromatin remodelling and PI3K pathway mutations in OCCC and demonstrate that these pathways converge on pro-tumorigenic cytokine signalling. We propose that ARID1A protects against inflammation-driven tumorigenesis.
Epithelial ovarian cancer (EOC) ranks as the fifth leading cause of cancer death among women\(^1\). EOC consists of four major histologic tumour subtypes (serous, clear-cell, endometrioid or mucinous) and originates from the coelomic epithelium, which is the precursor to the ovarian surface epithelium (OSE) and Müllerian ductal epithelium\(^2\). The salpinge or fallopian tube of the Müllerian epithelial anlage is the more definitive source of serous EOC\(^2\). Endometriotic explants originating from more distal sites in the Müllerian epithelial anlage may have some propensity to give rise to clear-cell or endometrioid EOC in humans, but this alternate mechanism of pathogenesis has not been thoroughly investigated\(^3\).

In addition to the histologic feature, each EOC subtype can be further classified into two distinct groups based on the disease progression, metastatic potential and molecular signature, with low-grade serous, clear-cell, mucinous and endometrioid subtypes being classified as Type I tumours and the high-grade serous subtype as Type II tumours\(^6\). In general, Type I tumours account for up to 25% of EOCs, are genomically stable and have lower metastatic potential than Type II tumours, which constitute most of the remaining EOC cases\(^6\). In addition, KRAS, BRAF, PIK3CA, PTEN and CTNNB1 (β-CATENIN) mutations have historically been associated with Type I tumours at varying frequencies, whereas Type II tumours are largely defined by high-frequency TRP53 (p53) mutations and chromosome instability\(^6\).

Therefore, EOC consists of a heterogeneous group of cancers, each with unique clinical challenges and biology.

**Figure 1 | A new Arid1a conditional allele to explore ARID1A tumour-suppressor function in vivo.** (a) Targeting scheme to insert two loxP sites flanking exon 5 and 6 of mouse ARID1A. (b) Representative Southern blot showing properly targeted embryonic stem cell clones. Insertion of loxP sites generates 5.6 kb EcoRV and 8.5 kb Apal genomic fragments. (c) PCR genotyping and quantitative, dual-colour fluorescent western blot of ARID1A (red) and βACTIN (green) protein expression in whole-embryo lysates from four Arid1a\(^{fl/+}\) and four Arid1a\(^{fl/+}; Sox2CRE\(^{cko}\)\) embryos. (d) Graph of quantitative western blot results depicting normalized protein levels. Significant differences based on the average normalized protein expression ± s.d. of four independent embryos were calculated using a two-tailed Student’s t-test (P values < 0.05 were considered significant). (e,f) Representative LacZ-stained, uninjected and AdCRE-injected (Gt)Rosa26lacZ ovaries. (g–i) Live-luminescence overlaid whole-mount images of an intrabursal AdCre-injected, (Gt)Rosa26LacZ female mouse. (j) Excised reproductive tract. Asterisks in h indicate wound staple.

OCCC is the most common Type I tumour, accounting for 5–25% of all EOC cases, with incidence varying between populations\(^9\)–\(^11\). Endometriosis is recognized as a significant risk factor for OCCC\(^12\)–\(^14\). Among all EOC subtypes, OCCC has the worst prognosis if diagnosed at an advanced stage of disease because of poor response rates to platinum-based chemotherapy\(^9\)–\(^10\),\(^15\)\(^\text{-}\)\(^16\). Consequently, survival rates for women with advanced stage OCCC are low\(^9\),\(^10\),\(^15\),\(^16\). Although OCCC is the second leading cause of death from ovarian cancer, the aetiology and pathogenesis of this devastating disease are poorly understood.

Recent genome-sequencing efforts support a strong genetic contribution to OCCC aetiology based on the discovery of high-frequency (up to 50%) ARID1A tumour mutations\(^17\),\(^18\). ARID1A is also somatically mutated in another Type I EOC, endometrioid carcinoma and several other gynaecologic cancers, including uterine clear-cell and endometrioid carcinomas, cervical carcinoma and uterine carcinosarcoma\(^19\),\(^20\). Additional tumour-sequencing studies have detected recurrent ARID1A mutations in several non-gynaecologic cancers and, taken together, components of the SWI/SNF complex are mutated in at least 20% of human cancers\(^19\),\(^21\)–\(^23\). ARID1A is a subunit within the SWI/SNF chromatin remodelling complex that facilitates target substrate recognition; however, its role in OCCC tumour initiation and progression has, yet, to be fully elucidated\(^24\).

Genetically engineered mouse models offer the opportunity to investigate the contribution of genetic factors to EOC aetiology.

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and pathogenesis. In this regard, early research using mouse models of EOC established causative roles for coexistent mutations in PTEN and KRAS or PTEN and WNT/β-CATENIN pathways in endometrioid EOC. Recently, Guan et al. reported a mouse model of endometrioid EOC involving coexistent mutations in PTEN and ARID1A. In this study, we describe a new mouse model that genetically and histologically resembles human OCCC and functionally implicates coexistent ARID1A and PIK3CA mutations in cancer causation. We further provide new mechanistic insight into OCCC pathogenesis by establishing a functional link between coexistent ARID1A–PIK3CA mutations and interleukin-6 (IL-6) signalling.

**Results**

**Generation of a novel Arid1a conditional allele.** To establish a functional role for ARID1A mutations in ovarian clear-cell tumorigenesis, we generated a novel Arid1a conditional allele (Arid1a\(^{fl}\)) by inserting loxP sites flanking exons 5 and 6 in ARID1A (Fig. 1a,b). Arid1a-null embryos die around gastrulation and thus are not amenable to efficient protein extraction. In light of its early embryonic lethality, we monoallelically inactivated ARID1A in embryonic tissues (via Sox2Cre) to verify our Arid1a\(^{fl}\) allele in the whole animal. Using this approach, we observed an expected 50% reduction in total protein from whole-embryo lysates (Fig. 1c,d). We next sought to inactivate ARID1A in the OSE. To do this, we employed the intrabursal AdCRE recombination system. Efficient CRE-mediated genetic recombination was consistently observed in the OSE and outer epithelial layers of tissues confined to the bursal space (Fig. 1f,h,i). We performed a series of intrabursal AdCRE injections on two independent cohorts of Arid1a\(^{fl}\) mice (\(n = 42\)) and followed them for \(\sim 1\) year. The mice remained tumour free with no significant changes in survival, other than age-related mortality, similar to the phenotypes reported by Guan et al. (Supplementary Fig. 1). The observation that ARID1A loss does not lead to tumour formation supports the notion that ARID1A mutations require additional mutational ‘hits’ in the OSE before tumorigenesis can ensue.

**Coexistent ARID1A–PIK3CA mutations initiate ovarian cancer.** To explore this further, we focused on the cancer proto-oncogene, PIK3CA (Phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha or p110\(\alpha\)), which also has high OCCC mutation rates. Recently, coexistent ARID1A–PIK3CA mutations were reported in gastric cancer. In addition, it has been observed that OCCC tumours harbouring PIK3CA mutations display a concomitant loss of ARID1A immunoreactivity, raising the possibility that ARID1A and PIK3CA mutations frequently co-occur in OCCC. To understand the degree of mutational co-occurrence, we compared the incidence of ARID1A and/or PIK3CA mutations in available tumour data sets curated in The Cancer Genome Atlas (TCGA) to OCCC tumour-sequencing data. We found that the majority of cancers carry genetic alterations in one of these genes; however, the rate of ARID1A–PIK3CA mutational co-occurrence (33%; \(P < 0.05\)) is highest in OCCC (Fig. 2a).

PIK3CA tumour mutations often result in an H1047R ‘hotspot’ substitution within the kinase domain, generating a catalytically active protein. PIK3CA amplification also frequently occurs in EOC. To investigate the effect of PIK3CA alterations on ovarian tumorigenesis, we used the CRE-inducible (Gt)Rosa26Pik3ca\(^{H1047R}\) allele to drive PIK3CA\(^{H1047R}\) expression from the Rosa26 locus. We observed multifocal sites of epithelial hyperplasia in the OSE of AdCRE-injected (Gt)Rosa26Pik3ca\(^{H1047R}\) mice, but no tumour formation (Table 1). Intrabursal AdCRE induction of PIK3CA\(^{H1047R}\), under the control of the endogenous promoter, also led to OSE hyperplasia in the mouse, suggesting that constitutive overexpression of PIK3CA\(^{H1047R}\) from the Rosa26 locus does not further augment this process.

We next evaluated the combinatorial effects of ARID1A loss and PIK3CA activation by performing AdCRE injections into the ovarian bursa of Arid1a\(^{fl}\);(Gt)Rosa26Pik3ca\(^{H1047R}\) mice. In stark contrast to the single mutants, Arid1a\(^{fl}\);(Gt)Rosa26Pik3ca\(^{H1047R}\) mice rapidly developed primary ovarian tumours and showed evidence of abdominal distension, warranting killing with a median latency period of 7.5 weeks post AdCRE injection (Fig. 2j–m). Evidence for tumour-specific ARID1A loss and PIK3CA\(^{H1047R}\) expression or PI3K pathway activation was detected by PCR amplification of tumour complementary DNA (cDNA) or by tumour immunohistochemistry (IHC) for ARID1A and phosphorylation of AKT at serine 473 (P-AKT S473; Fig. 2b–i). Increased morbidity coincided with the presentation of haemorrhagic ascites (Fig. 2j,l). Peritoneal metastases were detected in \(\sim 50\%\) of the double mutants, often residing near the contralateral, uninjected ovary (Fig. 2k, see Table 1). Distant metastases beyond the abdominal cavity were not evident on gross examination. We did not find evidence for genome instability in Arid1a\(^{fl}\);(Gt)Rosa26Pik3ca\(^{H1047R}\) tumour samples using mouse high-density SNP (single-nucleotide polymorphism) arrays (Supplementary Fig. 2).

**ARID1A haploinsufficiency does not lead to tumour formation.** Several studies point to a gene dose-dependent role for ARID1A in tumour suppression given the high incidence of heterozygous tumour mutations in cancer. To determine if an ARID1A haploinsufficiency coupled with PI3K pathway activation leads to ovarian cancer in our model, we performed AdCRE injections on Arid1a\(^{fl}\)\(^{+}\);(Gt)Rosa26Pik3ca\(^{H1047R}\) mice. OSE hyperplasia was observed in five out of the seven Arid1a\(^{fl}\)\(^{+}\);(Gt)Rosa26Pik3ca\(^{H1047R}\) mice, but evidence of tumour formation was not detected after the 11-week observation period, similar to the (Gt)Rosa26Pik3ca\(^{H1047R}\) only cohort (Fig. 2n,o, see Table 1). These data are consistent with the observation that most OCCC tumours carrying heterozygous mutations in ARID1A show the loss of ARID1A immunoreactivity and support the notion that additional mechanisms lead to the loss of ARID1A protein expression in OCCC. Thus, ARID1A likely follows the classical ‘two-hit’ model of tumour suppression in OCCC.

**Therapeutic PIK3CA inhibition prolongs animal survival.** We next sought to examine the role of continued PIK3CA activation in the progression of ARID1A-deficient ovarian tumours and determine whether therapeutic PIK3CA inhibition would inhibit tumour cell growth and improve animal survival. Primary EOC tumour cells were isolated from the exfoliated tumour cell aggregates present in ascites fluid of tumour-burdened Arid1a\(^{fl}\);(Gt)Rosa26Pik3ca\(^{H1047R}\) mice and subcultured using established methods (Fig. 3a). Isolated tumour cells displayed ARID1A loss and heightened PI3K signalling, as evidence by the loss of ARID1A immunoreactivity and increased P-AKT S473 levels compared with normal OSE cells (Fig. 3b). Administration of the pan-class I PI3K inhibitor, BKM120 (Buparlisib) led to a dose-dependent decrease in P-AKT S473 and concomitant reduction in tumour cell viability (IC\(_{50} = 0.96 \pm 0.25 \mu M\); Fig. 3c,d). We next administered chow-fed BKM120 to Arid1a\(^{fl}\);(Gt)Rosa26Pik3ca\(^{H1047R}\) mice for 3 weeks, starting at week 4 post AdCRE injection. The effectiveness of BKM120-dependent inhibition of PI3K signalling in chow-fed mice was accessed by tumour IHC...
Figure 2 | Concurrent ARID1A loss and PIK3CA activation leads to ovarian tumorigenesis in the mouse. (a) Plot of ARID1A and PIK3CA alterations across available TCGA data sets. Mutation or co-mutation frequencies are expressed as a percentage of all tumour samples for each cancer. A Fisher’s exact test was used to calculate the significance of association between ARID1A and PIK3CA mutations (*P value < 0.05). (b) Genotyping scheme to detect CRE-deleted (Arid1afl) allele in tumour genome DNA samples. RT-PCR was used to distinguish PIK3CA and transgenic (Gt)Rosa26Pik3caH1047R transcripts in tumour RNA samples. The H1047R mutation in (Gt)Rosa26Pik3caH1047R protects against MsiI digest of amplified tumour cDNA. (c) ARID1A loss or (Gt)Rosa26Pik3caH1047R transcripts were detected in tumour RNA samples by RT-PCR. Significant differences based on the average normalized mRNA expression ± s.d. between replicates of a control liver sample and replicates of three independent tumour samples were calculated using a two-tailed Student’s t-test (P values < 0.05 were considered significant). (d,e) ARID1A expression is observed in cells throughout the normal uterus and ovary by IHC. (e) ARID1A is expressed in the OSE of normal ovaries (arrowhead). (f) ARID1A expression is not observed in the tumours. (g) P-AKT S473 levels are low in the normal uterus. (h) P-AKT S473 in the normal ovary is highest in the OSE (arrowhead) and these levels are greatly increased in ovarian tumours (arrowhead). Asterisk in e denotes an oocyte. All sections processed for IHC were lightly counterstained with methyl green. (j-k) Morbid Arid1afl/fl; (Gt)Rosa26Pik3caH1047R mouse at killing with haemorrhagic ascites (inset), primary ovarian tumour of moderate size and bilateral tumour metastases (arrowheads). (l-m) Morbid Arid1afl/fl; (Gt)Rosa26Pik3caH1047R mouse at killing with haemorrhagic ascites (inset), large primary ovarian tumour and no visible metastases. The mice shown in j-m were killed at 7 and 9 weeks post AdCRE, respectively, because of visible ascitic fluid burden. (n,o) Arid1afl/fl; (Gt)Rosa26Pik3caH1047R mice at 11 weeks post AdCRE showing no evidence for tumour formation. In k,m, arrows indicate the AdCRE-injected ovary. In k,m,o, asterisks denote the uninjected, control ovary. ACC, adenocortical carcinoma; BLCA, bladder urothelial carcinoma; BRCA, breast invasive carcinoma; CESC, cervical squamous cell carcinoma and endocervical adenocarcinoma; COAD/READ, colon and rectum adenocarcinoma; GBM, glioblastoma multiforme; HNSC, head and neck squamous cell carcinoma; KICH, kidney chromophobe; KIRC, kidney renal clear-cell carcinoma; KIRP, kidney renal papillary cell carcinoma; LAML, acute myeloid leukaemia; LGG, brain lower-grade glioma; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; OV, ovarian serous cystadenocarcinoma; PAAD, pancreatic adenocarcinoma; PRAD, prostate adenocarcinoma; SKCM, skin cutaneous melanoma; STAD, stomach adenocarcinoma; THCA, thyroid carcinoma; UCEC, uterine corpus endometrial carcinoma; UCS, uterine carcinosarcoma.

Mouse ovarian tumours manifest OCCC-like histopathology. Cardinal histopathological features of human OCCC include cells with clear cytoplasm, stromal hyalinization, solid, papillary or tubulocystic tumour architectural patterns, and HNF1β immunoreactivity. The ovarian tumours observed in Arid1afl/fl; (Gt)Rosa26Pik3caH1047R mice were predominately solid in

for phosphorylation of the S6 ribosomal protein at serine 235/236 (P-S6; Fig 3e). BKM120 treatment led to reduced P-S6 levels and extended the median latency period by 3.5 weeks (Fig 3e,f). These data indicate that therapeutic PI3K inhibition promotes animal survival by inhibiting tumour cell growth, thus providing strong rationale for the use of PI3K inhibitors in OCCC treatment.
Tumour masses ranged from epithelium with distinct borders, and, in general, the tumours directly attached to the ovarian surface (Fig. 4a). We rarely hyalinized matrix. Solid clear-cell carcinomas were observed finely vacuolated cytoplasm were observed embedded in the human OCCC marker, HNF1α weeks (Supplementary Fig. 3). The tumours were also positive for early as 1 week following AdCRE injection, with exfoliated clumps of hobnail cells were evident on the ovarian surface as cell or hyalinized cores (Fig. 4i). OSE hyperplasia and small neoplastic cells with clear cytoplasm, prominent nucleoli and appearance with some papillary areas (Fig. 4a–d, see Table 1).

In human OCCC, neoplastic cells that line the luminal spaces often assume a ‘hobnail’ appearance, which crudely resembles a nucleus standing on the tip of a cytoplasmic stalk. Hobnail cells were often observed on the tumour periphery in regions where tumour cell exfoliation into luminal spaces was occurring (Fig. 4d.i,k). Exfoliated, metastatic tumour cell aggregates often consisted of hobnail-shaped cells organized around either clear-cell or hyalinized cores (Fig. 4i). OSE hyperplasia and small clumps of hobnail cells were evident on the ovarian surface as early as 1 week following AdCRE injection, with exfoliated tumour cell aggregates and clear-cell-like features apparent at 2 weeks (Supplementary Fig. 3). The tumours were also positive for the human OCCC marker, HNF1β, by IHC (Fig. 5b). Thus, mouse ovarian tumours harbouning coexistent ARID1A–PIK3CA mutations share histopathological features with human OCCC.

### Table 1 | Ovarian tumorigenesis requires concurrent ARID1A loss and PIK3CA activation.

|                      | *Arid1a<sup>fl/fl</sup>;<br> Gt(Rosa)26<sup>Pik3ca*H1047R</sup> | BKM120-treated *Arid1a<sup>fl/fl</sup>;<br> Gt(Rosa)26<sup>Pik3ca*H1047R</sup> | *Arid1a<sup>fl/fl</sup>;<br> Gt(Rosa)26<sup>Pik3ca*H1047R</sup> | *Arid1a<sup>fl/fl</sup>;<br> Gt(Rosa)26<sup>Pik3ca*H1047R</sup> | *Arid1a<sup>fl/fl</sup>;<br> Gt(Rosa)26<sup>Pik3ca*H1047R</sup> |
|----------------------|----------------------------------------------------------|----------------------------------------------------------|----------------------------------------------------------|----------------------------------------------------------|----------------------------------------------------------|
| OSE hyperplasia†     | —                                                        | —                                                        | —                                                        | 5/7 (71%)                                                | 0/6 (0%)                                                 | 4/5 (80%)                                                 |
| Ascites              | 12/30<sup>y,w</sup> (40%)                                | 6/10<sup>y</sup> (60%)                                    | 0/8 (0%)                                                | 0/4<sup>z</sup> (0%)                                      | 0/6 (0%)                                                 |
| Primary ovarian      | 23/30<sup>y,y</sup> (77%)                                 | 8/10<sup>y</sup> (80%)                                    | 0/8 (0%)                                                | 0/4<sup>z</sup> (0%)                                      | 0/6 (0%)                                                 |
| tumours (injected side) |                                          | —                                                        | —                                                        |  —                                                      |  —                                                      |
| Papillary/solid†     | 6/12 (50%)                                               | —                                                        | —                                                        |  —                                                      |  —                                                      |
| Tubulocystic†        | 2/12 (17%)                                               | —                                                        | —                                                        |  —                                                      |  —                                                      |
| Mixed†               | 4/12 (33%)                                               | —                                                        | —                                                        |  —                                                      |  —                                                      |
| Peritoneal metastases| 17/30<sup>y,y</sup> (57%)                               | 5/8<sup>z</sup> (63%)                                     | 0/8 (0%)                                                | 0/4<sup>z</sup> (0%)                                      | 0/6 (0%)                                                 |
| Contralateral ovarian metastases | 4/30<sup>y,y</sup> (13%)                   | 0/8 (0%)                                                | 0/8 (0%)                                                | 0/4<sup>z</sup> (0%)                                      | 0/6 (0%)                                                 |
| Contralateral peritoneal metastases | 15/30<sup>y,y</sup> (50%)                             | 4/8 (50%)                                               | 0/8 (0%)                                                | 0/4<sup>z</sup> (0%)                                      | 0/6 (0%)                                                 |

OSE, ovarian surface epithelium.

†Mice were euthanized and scored at the end of the 11-week observation period.

‡Scored by histology.

§Three mice found dead and not scored for ascites.

||Two mice found dead after treatment and not scored for ascites or primary tumours.

*Only morbid-euthanized mice were scored.

#Two mice had tumours within the body wall at incision site that were likely the result of leaky AdCRE and were not scored.

**Two mice found dead after treatment and not scored for ascites.

**One mouse had a very large stromer tumour that was likely a result of leaky AdCRE into the lumen of the oviduct or uterus and was not scored.

appearance with some papillary areas (Fig. 4a–d, see Table 1). Tubulocystic patterns were rarely observed (Fig. 4e, see Table 1). Neoplastic cells with clear cytoplasm, prominent nucleoli and pleomorphic nuclei were observed in the tumours (see high magnification in Fig. 4a–c’). Elongated spindle-shaped cells with finely vacuolated cytoplasm were observed embedded in hyalinized matrix. Solid clear-cell carcinomas were observed directly attached to the ovarian surface (Fig. 4a). We rarely observed tumour cells organized into bands of glandular epithelium with distinct borders, and, in general, the tumours appeared poorly differentiated and highly disorganized. Primary tumour masses ranged from ~100 mg to over 1 g (Fig. 4j). Differences in tumour size, metastatic potential or morbidity did not correlate with any particular histologic feature.

In human OCCC, neoplastic cells that line the luminal spaces often assume a ‘hobnail’ appearance, which crudely resembles a nucleus standing on the tip of a cytoplasmic stalk. Hobnail cells were often observed on the tumour periphery in regions where tumour cell exfoliation into luminal spaces was occurring (Fig. 4d.i,k). Exfoliated, metastatic tumour cell aggregates often consisted of hobnail-shaped cells organized around either clear-cell or hyalinized cores (Fig. 4i). OSE hyperplasia and small clumps of hobnail cells were evident on the ovarian surface as early as 1 week following AdCRE injection, with exfoliated tumour cell aggregates and clear-cell-like features apparent at 2 weeks (Supplementary Fig. 3). The tumours were also positive for the human OCCC marker, HNF1β, by IHC (Fig. 5b). Thus, mouse ovarian tumours harbouning coexistent ARID1A–PIK3CA mutations share histopathological features with human OCCC.

Tumour gene expression supports a role for IL-6 in OCCC. To further evaluate the relevance of our Arid1a<sup>fl/fl</sup>; Gt(Rosa)26<sup>Pik3ca*H1047R</sup> ovarian tumour model to human OCCC, we performed a series of cross-species gene expression comparisons using a well-characterized gene expression data set containing all four human EOC subtypes. Gene expression profiles of mouse primary tumour samples and matched normal (uninjected) ovaries were generated by microarray. Consistent with our genetic model, ARID1A and PIK3CA expression levels were inversely correlated in comparisons between tumour and normal ovary samples, with PIK3CA upregulation strongly correlated with ARID1A downregulation in the tumour samples (Supplementary Fig. 4). We verified our microarray gene expression results by reverse transcription-PCR (RT–PCR) validation of KRAS, TFIPI2, CDKN2A (P16), CDKN1A (P21), MUC16 (CA125) and VCAN expression (Supplementary Fig. 5).

We next identified all coordinately regulated genes in both mouse tumours and human OCCC, as compared with species-matched normal ovaries. In comparisons of both mouse and human data sets, the control ovary samples showed high levels of gene expression variation, suggesting tissue sampling differences or general interspecies-specific gene expression differences (Supplementary Fig. 6). Focusing on those genes that remained unchanged in normal mouse and human ovaries, we identified 584 coordinately regulated genes (272 upregulated; 312 downregulated) in mouse and human tumours (Supplementary Fig. 7). Mouse and human tumour gene expression patterns were enriched for genes involved in IL-6 signalling, immune system function, focal adhesion and regulation of the actin cytoskeleton, as compared with normal ovaries (Supplementary Figs 6 and 7).

Since many of the upregulated genes we identified in cross-species comparisons with normal ovaries are likely to be found in other EOC subtypes for example, CA125 or generally found across most cancers, we next sought to identify an OCCC-specific gene signature for use in subtype-specific gene expression comparisons with the mouse tumours. To do this, we compiled a more refined ‘discriminant’ gene list for each subtype (for example, clear-cell versus all other EOC subtypes). We then compared the normalized raw expression values for each discriminant gene across all of the mouse tumour and human EOC samples. Using this analysis, we found that the gene expression patterns for mouse tumour and human OCCC samples were not statistically different (P = 0.6), suggesting that the OCCC-specific gene expression patterns are conserved between the two species (Fig. 5c). Several well-known markers of OCCC were identified as being highly expressed in the mouse tumours, including IL-6, STAT3, VCAN and HIF1α, further supporting a role for heightened IL-6 signalling in OCCC tumour pathogenesis.

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To further assess the similarities between our genetic model and human OCCC, we compared the mouse tumour-specific gene expression profiles for our Arid1afl/fl; (Gt)Rosa26Pik3ca*H1047R mouse model to those recently reported for the Arid1afl/fl; Ptenfl/fl mouse model by Guan et al.26, which is genetically similar endometrioid EOC. Both models relied on the intrabursal AdCRE injection method for mutation induction. We applied principal component analysis using the normalized expression values for all genes in the tumour data sets for each mouse model. We found that each tumour sample clustered based on the genotype or mutational pattern of the respective genetic model, with the exception of one Arid1afl/fl; Ptenfl/fl tumour (Supplementary Fig. 8). These findings are consistent with the marked phenotypic differences observed in our Arid1afl/fl; (Gt)Rosa26Pik3ca*H1047R tumours when compared with the phenotypes reported for Arid1afl/fl; Ptenfl/fl tumours by Guan et al.26.
Autocrine IL-6 signalling promotes tumour cell growth. IL-6 is an inflammatory cytokine that triggers JAK/STAT3 signalling and has prominent roles in tumour cell growth and differentiation. High levels of circulating IL-6 in cancer patients are associated with a poor prognosis. In addition to its increased expression in OCCC tumours, IL-6 has been detected in patient serum and tumour-derived cell lines, which has led to its use as a therapeutic target in OCCC. In support of our cross-species microarray comparisons, we verified IL-6 expression in primary tumours and peritoneal metastases of Arid1afl/fl(Gt)Rosa26Pik3caH1047R mice by RT–PCR (Fig. 6b). We also observed high levels of secreted IL-6 in the body fluids of tumour-burdened mice, with >2,000 pg ml⁻¹ of IL-6 in ascitic fluid aspirates (Fig. 6c,d). Consistent with this, strong IL-6 immunoreactivity was also observed in the mouse tumours by IHC (Fig. 6e).

To understand the role of IL-6 in OCCC pathogenesis, we treated primary Arid1afl/fl(Gt)Rosa26Pik3caH1047R mouse ascites-derived tumour cells, isolated as described above, with anti-IL-6-neutralizing antibodies. Anti-IL-6 treatment reduced the tumour cell viability and downregulated the phosphorylation of STAT3 at tyrosine 705 (P-STAT3 Y705), suggesting that autocrine IL-6-STAT3 signalling promotes tumour cell growth (Fig. 6f,g). Next we generated primary mouse tumour cell lines that stably express IL-6 short hairpin RNAs (shRNA) for use in tumour growth assays (Fig. 6h,i). IL-6 shRNA-expressing cells showed reduced growth rates in culture, and media from IL-6 shRNA-expressing cells contributed to reduced tumour growth (Fig. 6j, k). The rate of tumourgraft growth for IL-6 shRNA-expressing mouse cells onto the flanks of immunodeficient mice. Remarkably, the ascitic tumour cell grafts were indistinguishable from the ovarian tumours with regards to histopathological features (for example, clear cytoplasm), despite being maintained in tissue culture and further grown under the skin of immunodeficient nude mice. The ascitic tumour cell grafts were significantly less than control shRNA-expressing cells, further indicating that autocrine IL-6 signalling contributes to OCCC pathogenesis by promoting tumour cell growth (Fig. 6m).
IL-6 induction requires coexistent ARID1A–PIK3CA mutations.

To determine if coexistent ARID1A–PIK3CA mutations are required for IL-6 induction, we utilized an AdCRE-inducible primary OSE cell culture model. IL-6 expression was measured following AdCRE infection of primary OSE cells isolated from mice carrying all mutant allele combinations. Loss of ARID1A or PIK3CA activation alone led to significant increases in IL-6 expression by RT–PCR and enzyme-linked immunosorbent assay (ELISA), and IL-6 induction was further enhanced when ARID1A and PIK3CA were co-mutated together in Arid1aβ/β;GtRosa26Pik3caH1047R cells (Fig. 7a,b). Consistent with this, P-STAT3 Y705 levels also correlated with similar increases in IL-6 activity in AdCRE-treated OSE cells (Fig. 7c). ARID1A loss in AdCRE-treated Arid1aβ/β or Arid1aβ/β;GtRosa26Pik3caH1047R OSE cells did not further enhance PI3K pathway activity (Fig. 7c). We found that Arid1aβ/β;GtRosa26Pik3caH1047R OSE cell proliferation was suppressed by treatment with anti-IL-6-neutralizing antibodies following mutation induction with AdCRE, and that recombiant IL-6–6 enhances normal OSE cell proliferation and IL-6-STAT3 signalling, further suggesting that IL-6 is both necessary and sufficient for tumour cell growth (Fig. 7d–f). To determine if ARID1A is bound at the IL-6 promoter under repressed conditions, we performed a series of ARID1A chromatin immunoprecipitation (ChIP) experiments in primary OSE cells. We used Arid1aβ/β;GtRosa26Pik3caH1047R OSE cells for these experiments so that AdCRE-treated or ARID1A-depleted cells could be used as a control for ARID1A immunoprecipitation specificity. In AdControl-treated cells, increased ARID1A occupancy was observed at sites near the IL-6 promoter, but not at distal upstream or downstream sites (Fig. 7g). ARID1A occupancy at this site decreased in a manner consistent with ARID1A depletion following AdCRE infection. This data indicate that coexistent ARID1A–PIK3CA mutations lead to IL-6 upregulation.

Discussion

Our data support a genetic epistasis model wherein ARID1A and PIK3CA mutations cooperate and ovarian cancer can arise only when these genes are co-mutated in the mouse OSE. The short latency period, lack of copy-number variation and OCCC-like histological and molecular features we observed are also consistent with coexistent ARID1A–PIK3CA mutations being a major driver of OCCC in humans. Our results suggest that high levels of tumour cell-derived IL-6 are promoting tumour growth in OCCC. Indeed, the top pathway we identified in common between species is comprised of genes involved in this pathway, suggesting that IL-6-STAT3 signalling is inherent to OCCC biology. Hence, IL-6 serves as both a molecular marker and a prospective therapeutic target in OCCC. Therefore, anti-IL-6 antibody (Siltuximab) therapy may prove to be a safe and effective treatment strategy for OCCC patients. More broadly, our findings further demonstrate that the continued identification of tumour mutations (that is, TCGA projects) will allow us to identify potential functional relationships among recurrent mutational patterns or cancer pathways that would have otherwise gone unnoticed.

The OSE-derived tumour phenotypes we observed in our Arid1aβ/β;GtRosa26Pik3caH1047R mouse model of OCCC greatly contrasted with those reported by Guan et al.26 for Arid1aβ/β;Ptenβ/β mice. For example, all of the tumour-burdened Arid1aβ/β;GtRosa26Pik3caH1047R mice were morbid or succumbed to death within 3 months after AdCRE injection, whereas Guan et al.26 reported tumours in a subset of Arid1aβ/β;Ptenβ/β mice after 6 months, indicating stark differences in tumour latency or survival. Molecular differences were evident in tumour gene expression comparisons between the two mouse models, as each gene expression data set parsed the majority of tumour samples based on its respective mutational pattern or genotype. These data point to potential functional differences between ARID1A–PIK3CA and ARID1A–PTEN mutational patterns in Type 1 EOCs and support the idea of a possible PI3K-AKT-mTOR-independent role for PTEN in endometroid EOC.26 Alternatively, it remains possible that clear-cell tumorigenesis is driven, in part, by an alternative AKT-independent mechanism downstream of oncogenic PIK3CA mutations.45,46 Additional pharmacological studies targeting downstream effectors or alternative signalling pathway components will further address potential PI3K pathway-independent mechanisms of endometroid versus clear-cell tumorigenesis.
**Figure 6** | Tumour-derived IL-6 promotes OCCC tumour cell growth and survival. (a) Top OCCC-specific genes in common between mouse and human tumours. Top six Ingenuity Pathway Analysis (IPA) and MSigDB (GSEA) predictions of the top upregulated genes are included. (b) RT-PCR validation of IL-6 expression in primary tumours and peritoneal metastases. Significant differences based on average normalized mRNA expression ± s.d. between peritoneal metastases or primary tumours and matched uninjected ovaries were calculated using a two-tailed Student’s t-test. (c,d) Mouse IL-6 levels in the serum and ascitic fluid Andtdfl/fl(Gt)Rosa26Pik3caH1047R mice, as measured by anti-IL-6 ELISAs. Significant differences based on the average protein concentration ± s.d. between wild-type versus Andtdfl/fl(Gt)Rosa26Pik3caH1047R mice were calculated using a two-tailed Student’s t-test. (e) IL-6 expression in an Andtdfl/fl(Gt)Rosa26Pik3caH1047R tumour by IHC. (f) MTT absorbance values plotted with log antibody concentration (μg ml⁻¹) for non-specific rat IgG-treated (control) or rat anti-mouse IL-6-treated Andtdfl/fl(Gt)Rosa26Pik3caH1047R ascitic tumour cells after 96 h of treatment. Plot represents the average absorbance value ± s.d. for treatment performed on three independent cell lines. Significant differences between control- and anti-IL-6-treated cells were calculated using a two-tailed Student’s t-test (*significant P value <0.05). (g) Primary ascitic tumour cells were treated 10, 1, 0.01 or 0 μg ml⁻¹ rat anti-mouse IL-6 or non-specific Rat IgG-treated (control). (h) IL-6 expression in Andtdfl/fl(Gt)Rosa26Pik3caH1047R ascitic tumour cells stably expressing control shRNAs or IL-6 shRNAs by ELISA and western blot. Each replicate represents in stable pool of IL-6 shRNA-expressing cells from three independently isolated tumour cell lines. Significant differences based on the average protein concentration ± s.d. between control and IL-6 shRNA were calculated using a two-tailed Student’s t-test. (i) MTT absorbance values plotted over time for control shRNA-, IL-6 shRNA- or IL-6 shRNA-expressing cells supplemented with 10 ng ml⁻¹ IL-6. Cells were plated at 2 x 10⁴ cells ml⁻¹ at hour 0, then MTT measurements were taken every 24 h for a total of 96 h. Significant differences between control- and IL-6 shRNA-expressing cells were calculated using a two-tailed Student’s t-test (*significant P value <0.05). (k) Whole-mount images of control- and IL-6 shRNA tumourgrafts (arrowheads) on the right flank of nude mice and images of corresponding haematoxylin and eosin-stained tumourgraft sections. (m) Plot of growth rates for control- and IL-6 shRNA-expressing tumourgrafts over 21 days of measurement. Significant differences between control- and IL-6 shRNA tumourgraft growth rates were calculated using a two-tailed Student’s t-test. Only P values <0.05 were considered significant. MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.
Several theories on the origins of OCCC have been proposed. Our data strongly suggest that the OSE harbour a cancer-prone cell population that gives rise to OCCC-like tumours in the mouse, which is consistent with the classical view of EOC pathogenesis. Nonetheless, our mouse model does not address the role of distal endometriotic explants or other putative precursor lesions in OCCC pathogenesis. Specifically targeting the ARID1A–PIK3CA mutational pattern to other tumour-prone cell population that gives rise to OCCC-like tumours in the mouse, which is consistent with the classical view of EOC pathogenesis. Nonetheless, our mouse model does not address the role of distal endometriotic explants or other putative precursor lesions in OCCC pathogenesis. Specifically targeting the ARID1A–PIK3CA mutational pattern to other tumour-prone cell population that gives rise to OCCC-like tumours in the mouse, which is consistent with the classical view of EOC pathogenesis. Nonetheless, our mouse model does not address the role of distal endometriotic explants or other putative precursor lesions in OCCC pathogenesis.

Inflammation of coelomic epithelial cell derivatives in the female reproductive tract is thought to be a major contributor to malignant transformation in endometriosis-associated EOC, like OC1.5. According to inflammatory mediators, aberrant immune modulation or irregular reproductive hormone levels are thought to play a major role in malignant transformation of OC1.5. Inflammatory cytokine signalling pathways, like IL-6, may be part of the natural inflammatory repair process accompanying each ovarian and menstrual cycle that, when left unchecked, promotes unregulated epithelial cell growth. In support of this, our data demonstrate that OSE cells are competent to respond to and upregulate IL-6. Therefore, the incomplete resolution of epithelial cell inflammation during these repair processes may represent a common mechanism underlying OCCC tumorigenesis, irrespective of mutational pattern.

The identification of IL-6 as a physiological target of ARID1A tumour-suppressor activity in our OCCC tumour model raises many important questions regarding the role of
ARID1A-containing SWI/SNF complexes in normal coelomic epithelial cell homeostasis. Indeed, ARID1A mutations have been identified in a wide variety of cancers originating from coelomic epithelial derivatives throughout the gynaecologic tract. Moreover, loss of ARID1A immunoreactivity is observed in endometriosis, a disease intimately associated with inflammation28,35. Endometriosis is also characterized by increased IL-6 expression, but the relationship between ARID1A loss and IL-6 upregulation in endometrial tissue is unknown31,32. Thus, ARID1A may regulate coelomic epithelial cell homeostasis and prevent tumorigenic conversion by negatively regulating the inflammatory programs required for normal tissue repair during the female reproductive cycle. It will be interesting to know if reproductive hormones influence ARID1A activity in this regard. Additional work on the propensity of ARID1A mutant epithelial cells to undergo malignant transformation in response to inflammatory ‘insults’ or the role of key inflammatory response pathways in this process will be required to address these hypotheses. Thus, the loss of ARID1A-containing SWI/SNF complexes might expose the ‘darker’, pro-tumorigenic side of inflammation.

How do oncogenic PI3K signalling mutations contribute to this process or lead to increased IL-6 expression? Positive regulators of IL-6 include JAK/STAT, EGFR/HER2, RAS/RAF/MEK, TNF/TLR1/IL1 or RAS/ERK oncogenic pathways and AP-1, SP1, NF-κB, C/EBP, STAT3, and CREB transcription factors.53,54,55 We propose that oncogenic PIK3CAH1047R mutations act like inflammatory ‘insults’ and drive the signalling loop that sustains high levels of IL-6 production in the absence of negative regulation by ARID1A (Fig. 7h). Additional studies focusing on the intricacies of this feed-forward signalling loop and the potential crosstalk mechanisms occurring among the various pathways that sustain it will further explain why coexistent ARID1A-PIK3CA mutations are found in a wide variety of human cancers.

**Methods**

**Generation of the Arid1a conditional mouse allele.** The Arid1a conditional mutant mouse allele was engineered using the bacterial artificial chromosome recombineering methods developed by Liu et al.54 Correctly targeted mouse embryonic stem cells were identified by Southern blotting. The Neo selection cassette used for targeting was removed by FLP-mediated recombination55. Targeted embryonic stem cells were injected into C57Bl6 blastocysts and implanted into pseudopregnant females. Germline animals were detected by coat colour and drug treatments were performed in accordance with protocols approved by the University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee.

**Reverse transcription-PCR.** Total RNA was extracted from pulverized tumour samples or ovarian epithelial cells using the TRIzol method (Invitrogen), followed by DNase I cleanup step and on-column DNA digestion using the RNA-prep mini prep kit (Qiagen) according to the manufacturer’s instructions. To analyse ARID1A and (G/C)626P6a/c1/H1047R gene transcript levels, real-time quantitative PCR was performed using Ssofast PCR master mix (BioRad) and a CFX96 thermocycler (BioRad) with the following gene-specific primers: ARID1A (F) 5'-CTGAAAAGAAGGCCGCATCCT-3', ARID1A (R) 5'-CCACACTCCGAATCTC-3', R26eson1 (F) 5'-CTAGTGAAGGAGTGCAAGCTCT-3', PIK3CA-dNA (R) 5'-AATTCTCTTGGTGAGCAGGCTCT-3'. The expression was calculated from three independent experiments using ΔΔCT methods. Expression levels were normalized to JACTIN. Statistical differences were detected using a two-tailed Student’s t-test. The following H1047R spanning PCR primer pair was used to distinguish endogenous PIK3CA from the conditional mouse allele: (F) 5'-AAGAGGATCAGTGAAATTCATT-3', (R) 5'-GGGAACAGTTGCTTGCG-3'. The PCR products were digested with MsiI and resolved by 2% Tris-Borate-EDTA agarose gel electrophoresis.

**Western blotting.** Western blots were performed using the following antibody dilutions and standard chemiluminescent detection methods or Li-Cor Bioscience Odyssey fluorescent western blotting reagents: 1:1,000 ARID1A (A301-041A, Bethyl Labs.), 1:500 IL-6 (ab672, Abcam), 1:2,000 Phospho-AKT Ser473 (4060, Cell Signaling), 1:1,000 AKT (4691, Cell Signaling), 1:100 STAT3 (sc-482, Santa Cruz Biotechnology), 1:1,000 Phospho-STAT3 Tyr705 (9145, Cell Signaling) and 1:5,000 JACTIN (Abcam). Membranes were blocked in 1× PBS supplemented with 5% (w/v) non-fat dry milk (Blotto B) or 1× Tris-buffered saline (pH 7.6) supplemented with 5% (w/v) non-fat dry milk, 1% (w/v) BSA and 0.05% (v/v) Tween-20 (Blotto B) or 1× Tris-buffered saline (pH 7.6) supplemented with 5% (w/v) BSA. Western blot images have been cropped for presentation. Full-size western blot images with antibody dilutions and blocking buffer conditions are presented in Supplementary Figs 9 and 10.

**Enzyme-linked immunosorbent assay.** IL-6 ELISAs were performed using the OptEia mouse or IL-6 kit reagents (BD Bioscience) according to the manufacturer’s instructions. ELISA measurements were normalized to total cellular protein.

**Copy-number and TCGA computational analyses.** Tumour and matched control DNA samples were processed for hybridization on the High Sensitivity Mouse Universal Genotyping SNP Array (MegaMUGA, Neogen) using published methods59. Copy-number analysis was performed using ASCAT59, ARID1A
and/or PIK3CA TCGA mutation frequency was identified by querying the cBioPortal on 2014-10-21 using the R API60,61.

Microarray and cross-species gene expression comparisons. Total RNA was isolated from nine primary tumour specimens and nine matched normal (unjected) oocytes as described above. Total RNA (250 ng) was used to synthesize fragmented and labelled sense-strand cDNA and hybridize onto Affymetrix Mouse Gene 2.1 ST peg plate arrays. The mouse data were uploaded to the Gene Expression Omnibus (GEO) under the accession number: GSE57380. Affymetrix CEL files were analyzed using the Robust Multi-chip Average normalization method62. Expression changes (tumour versus control ovary) were determined using Linear Models for Microarray Data Analysis63 or Significance Analysis of Microarray analysis64. Probes with a false discovery rate of 0% were considered statistically significant. For mouse-human cross-species comparisons, expression data for human OCCC and normal oocytes were downloaded from GEO under the accession number GSE6008 (ref. 39). Heat maps and dendrograms were generated with Java TreeView or R (www.R-project.org). Histological subtype-specific gene signatures were derived by first identifying differentially expressed genes between human normal tissue and cancer tissue in GSE6008. Next, differentially expressed genes were identified comparing each histological subtype to all other histological subtypes. We considered only the tumour versus normal comparison and in a particular subtype were removed because these are not specific to a histological subtype and may reflect generic pan-cancer gene expression patterns. To compare human and mouse samples from these gene sets, we first determined Z-scores for each gene within the mouse or human. We combined these Z-scores and Z-scored the combined data using all human and mouse tumour samples in GSE6008 and this study. Pathways analysis was performed using the GSEA Molecular Signatures Database (MSigDB)65,66 or Ingenuity Pathway Analysis (Qiagen).

Ascites-derived tumour cells and OSE cell culture. Primary ascites-derived ovarian cancer cells were isolated and grown as previously described67. To facilitate complete red blood cell lysis, the cells were incubated and washed in deionized H2O before resuspension in growth medium. Mouse OSE cells were isolated and cultured as previously described68. To induce CRE-mediated recombination in OSE cells, the cells were infected with AdCRE or AdControl particles (obtained from the University of Iowa Gene Transfer Core) at an MOI of 200 for 2 h in serum-free media. Infected OSE cells were then placed in normal growth medium and allowed to recover for 3 days before further manipulation. BKM120 (Chemietek), low-endotoxin rat anti-mouse IL-6 neutralization antibody (R&D Systems, MAB406), non-specific rat IgG1, isotype control antibody (R&D Systems, MAB005) or recombinant mouse IL-6 protein (R&D Systems, 406-ML-005) were reconstituted according to the manufacturers’ instructions and used as described in the text. For all antibody neutralization experiments, the cells were refed daily with the appropriate concentrations of antibody. Primary ascites-derived ovarian tumour cells were stably transduced with Mission pLKO.1-puro IL-6 shRNA (Hairpin sequence: 5’-CGCGGACAGAGGACGAGAAGA-3’/5’-CTATCGTTCTTGGTGAGGCTCCAGAGC-3’/5’-GACTTCGTGTTTTG-3’/Sigma, TRCN0000006751) or non-target control shRNA (Sigma, SC1016V) Lentiviral transduction particles according to the manufacturer’s instructions. The MTT assay kit (Roche) was used to measure cell viability. Ascites-derived tumour cells and OSE cells were passage using Accutase (Invitrogen). Half-maximum inhibitory concentrations (IC50) were calculated by curve-fitting using Kaleidagraph software (Synergy).

Tumour grafts. Primary ascites-derived ovarian tumour cells expressing control- or IL-6 shRNAs were harvested by Accutase digestion and washed in 1 × PBS. The cells were then injected subcutaneously into the right flanks of immunodeficient mice at a concentration of 5 × 106 cells ml−1 in a 1:1 (v/v) solution of 1 × PBS and Cultrex basement membrane extract (3432-005-01, Trevigen). Tumour graft volumes were calculated using biweekly caliper-based measurements and growth rates were based on these volumetric measurements. All tumour grafts were performed in accordance with protocols approved by the University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee.

Histology and IHC. For indirect methods of slides, 10% neutral-buffered formalin-fixed paraffin sections were processed for heat-based antigen unmasking in 10 mM sodium citrate (pH 6.0). Sections were incubated with the following antibody dilutions: 1:50 ARID1A (Clone PS3G, sc-32761, Santa Cruz Biotechnology), 1:100 Cytokeratin 8 (TROMAI, DSHB), 1:50 phospho-AKT Ser235/236 (4060, Cell Signaling), 1:50 phospho-ARID1A (Clone PSG3, sc-32761, Santa Cruz Biotechnology), 1:100 Cytokeratin 8 (TROMAI, DSHB), 1:50 phospho-AKT Ser235/236 IHC. Secondary detection was performed using Vectastain ABC System antibody diluent (8112, Cell Signaling) and SignalSlide AKT controls (8115, Cell Signaling). Sections were incubated with the following antibody dilutions: 1:50 phospho-AKT Ser473 (4060, Cell Signaling), 1:50 phospho-ARID1A (Clone PSG3, sc-32761, Santa Cruz Biotechnology), 1:100 Cytokeratin 8 (TROMAI, DSHB), 1:50 phospho-AKT Ser235/236 IHC. Sections processed for IHC were lightly counterstained with haematoxylin. Histological staining

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Author contributions
R.L.C. conceived this study, developed the hypothesis and wrote the manuscript with supervision and gracious support from T.M. R.L.C., J.S.D., J.C.S., D.B.D., F.P.-M.d.V., W.Y.K. and T.M. contributed to the experimental design. R.L.C., J.S.D., J.C.S., D.S., D.Y. and J.X. performed the experiments. J.R.R. and J.P.D. performed the computational analysis with conceptual advice from M.D.W. and J.S. All the authors commented on the manuscript.

Additional information
Accession codes: Microarray gene expression data sets generated in this study were deposited in the Gene Expression Omnibus (GEO) repository under the accession code GSE57380.

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