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Review

The role of the orphan nuclear receptor COUP-TFII in tumorigenesis

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The chicken ovalbumin upstream promoter transcription factors (COUP-TFs), members of the nuclear receptor superfamily, consist of two highly homologous subtypes, COUP-TFI (EAR-3, NR2F1) and COUP-TFII (ARP-1, NR2F2). They are referred to as orphan receptors because the COUP-TF ligands have yet to be identified. Since the discovery of COUP-TFs in 1986, extensive studies have demonstrated their crucial functions in a variety of developmental processes, such as organogenesis, angiogenesis, and metabolic homeostasis. Recently, emerging evidence has highlighted that COUP-TFs, specifically COUP-TFII, play important roles in tumorigenesis. In this review, we will discuss the critical functions of COUP-TFII in the development of the tumor microenvironment, the progression of various cancers, and its underlying mechanisms.

Keywords: nuclear receptor; COUP-TFII; tumorigenesis; prostate cancer; breast cancer

Introduction

The chicken ovalbumin upstream promoter transcriptional factors (COUP-TFs) belong to the steroid/thyroid hormone receptor superfamily and were first cloned in 1986[1–3]. Because the ligands of the COUP-TFs have not yet been identified, the COUP-TFs are referred to as orphan nuclear receptors. In vertebrates, two COUP-TF homologues have been identified, COUP-TFI (EAR3)[4, 5] and COUP-TFII (ARP-1)[6, 7], also known as nuclear receptor 2 family 1 and 2 (NR2F1 and 2). The COUP-TF family has a highly conserved modular structure that is composed of an N-terminal DNA binding domain (DBD) and a putative C-terminal ligand-binding domain (LBD). The COUP-TF DBD and LBD share 98% and 96% amino acid homology, respectively. Both receptors were highly conserved during evolution; human COUP-TFs share almost 95% homology with other vertebrate and invertebrate homologous proteins, suggesting that COUP-TFs could be primordial members of the NR family[8].

COUP-TFs have been shown to play important roles in many developmental processes. Both COUP-TFs are expressed during early embryonic development in mice and their expression is reduced shortly after birth. COUP-TFI is mainly expressed in the developing peripheral and central nervous systems, while COUP-TFII is detected in the mesenchyme of various organs and the developing vasculature[9]. COUP-TFI deletion in mice results in perinatal lethality mainly due to defects in central nervous system. In contrast, COUP-TFII null mice exhibit vascular abnormalities in the head, spine and heart, and die around embryonic day 10 (E10)[9]. Consistent with these results, recent studies using exome sequencing in human patients revealed that COUP-TFI mutations are associated with cerebral visual impairment (CVI)[10], while variants of COUP-TFII cause congenital heart defect (CHD)[11]. This research highlights the crucial role of COUP-TFI in neuronal development and COUP-TFII in organogenesis.

Because COUP-TFs are expressed at very low basal levels in adult mice, deletion of COUP-TFs in adults yielded no discernible phenotypes, except in reproduction with respect to the loss of COUP-TFII. In contrast, emerging evidence suggests that ectopic expression of COUP-TFs, specifically COUP-TFII, might play a critical role in the development of diseases, such as cancer and heart disease. In this review, we will focus on the functional roles of COUP-TFII and its underlying mechanisms in tumorigenesis.

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COUP-TFII in the tumour microenvironment

It is well recognised that angiogenesis and lymphangiogenesis are required for tumour progression, invasion, and metastasis. The blood and lymphatic vessels deliver nutrients and oxygen essential for tumour growth and provide access for metastases to distant sites. Without persistent vessel formation, tumour cells will undergo apoptosis or become necrotic[12, 13]. Therefore, a better understanding of the mechanisms of how tumours control this angiogenic switch will contribute to the development of new therapeutic strategies.

Our previous studies have revealed that COUP-TFII plays important roles in angiogenesis and lymphangiogenesis during normal mouse development. For example, *COUP-TFII* knockout mice have defects in atrial and vascular development and die around E10[14]; at a later developmental stage, conditional ablation of *COUP-TFII* in mice results in the malformation of the primitive lymphatic sacs and the lymphatic vessels[15]. To further investigate the roles of COUP-TFII in adult and pathologic angiogenesis, we used a TAM-inducible Cre-loxP system to knockout *COUP-TFII* in adult mice. The *COUP-TFII* ablation in the adult mice severely compromised neoangiogenesis and suppressed tumour growth in xenograft mouse models[16]. Moreover, inactivation of COUP-TFII in multiple spontaneous tumour models also impaired tumour progression and metastasis by repressing angiogenesis[16, 17]. This indicates that COUP-TFII serves as a major regulator to control angiogenesis within the tumour microenvironment. Further investigations revealed that COUP-TFII regulates two major angiogenic signals, Ang-1/Tie2 and VEGF/VEGFR-2. First, COUP-TFII positively regulates Ang-1 expression in the pericyte[16]. Ang-1 is a paracrine ligand for endothelium-specific tyrosine kinase receptor Tie2. Ang-1/Tie2 signalling plays prominent roles in both the maintenance of vascular quiescence and the promotion of angiogenesis[18]. COUP-TFII directly binds to the promoter of Ang-1 and enhances the expression of Ang-1, therefore activating the Ang-1/Tie-2 pathway to promote vessel remodelling[16]. Second, COUP-TFII represses the expression of VEGFR-1 in endothelial cells[17]. VEGFR-1 acts as a decoy receptor, sequestering VEGF from VEGFR-2 binding[19]. Thus, repression of VEGFR-1 by COUP-TFII enhances VEGF/VEGFR-2 signalling and promotes blood endothelial cell proliferation and sprouting. We also found that COUP-TFII upregulates E2F1 and downregulates Notch signalling[20, 21], and both have been shown to modulate angiogenesis via the VEGF/VEGFR axis[22, 23]. There might be a complicated interplay network between COUP-TFII, E2F1, Notch signalling and VEGF/VEGFR signalling to coordinate angiogenesis within the tumour microenvironment. However, the detailed mechanisms of how COUP-TFII conducts those signals needs to be further elucidated.

In addition to angiogenesis, accumulated evidence also suggests that COUP-TFII modulates tumour lymphangiogenesis. In our breast and pancreatic cancer mouse models, depletion of *COUP-TFII* impaired lymphangiogenesis and inhibited lymph node metastasis[15, 17]. Consistent with our results, previous reports showed that the COUP-TFII expression levels were positively correlated with lymph node metastasis in 119 breast tumour patients[24]. Based on the current understanding, multiple pathways might be involved in the modulation of COUP-TFII in tumour lymphangiogenesis. For example, the Ang-1/Tie2 axis might contribute to this progress. Because Tie2 is expressed both in lymphatic (LECs) and blood (BECs) endothelial cells[25], upregulation of Ang-1 by COUP-TFII in the pericyte also promotes LEC proliferation and lymphatic vascular remodelling via Tie2 signalling. The inactivation of COUP-TFII also severely inhibits the expression of VEGFR-3 and Nrp2[15], the co-receptor of VEGF-C in LECs. Together with previous reports, which showed that knockdown of COUP-TFII reduces the expression of VEGF-C in breast cancer cells[24], these results suggest that COUP-TFII controls tumour lymphangiogenesis by modulating the VEGF-C/VEGFR-3 pathway.

As depicted in Figure 1, COUP-TFII is a central regulator for both tumour angiogenesis and lymphangiogenesis by regulating VEGF/VEGFR, Ang-1/Tie2 and other pathways involved in manipulating the tumour microenvironment. These studies highlight the fact that COUP-TFII could serve as a potential target for anticancer interventions.

COUP-TFII in prostate cancer

Aside from its function within the tumour microenvironment, recent studies have also revealed that COUP-TFII promotes prostate cancer progression by directly regulating tumour growth. COUP-TFII immunostaining results in a prostate tumour tissue array indicated that COUP-TFII expression is higher in prostate tumour cells compared with the adjacent normal prostate epithelium. Among 407 patient specimens, approximately 60% of the cancer specimens exhibited intermediate to intense nuclear COUP-TFII staining, whereas only 5% of normal prostate epithelium cells were COUP-TFII positive[26]. Moreover, higher levels of COUP-TFII expression correlated with earlier tumour recurrences after radical prostatectomy[26]. Consistent with these results, data analysis from the Oncomine dataset also showed that COUP-TFII expression is higher in prostate cancer cells and further increased in metastatic prostate cancers[27, 28], indicating that COUP-TFII may promote prostate tumorigenesis. In support of this notion, prostate-specific ablation of the *COUP-TFII* gene in *Pten*-null mice prevented the progression of low-grade to advanced PIN lesions and adenocarcinomas and diminished cancer cell metastasis. Conversely, overexpression of COUP-TFII in *Pten*-null mice accelerated the progression of PIN to invasive adenocarcinoma, and enhanced metastasis to the lung and lymph nodes[26]. Interestingly, conditional depletion or overexpression of COUP-TFII in the prostate epithelium could not impair angiogenesis, suggesting that COUP-TFII might regulate other pathways beyond angiogenesis to promote prostate cancer progression.

Pten loss has been shown to activate TGF-β signalling and induce a growth barrier to constrain prostate cancer growth in the premalignant state[29]. The expression of COUP-TFII is reversely correlated with TGF-β signalling in the pros-
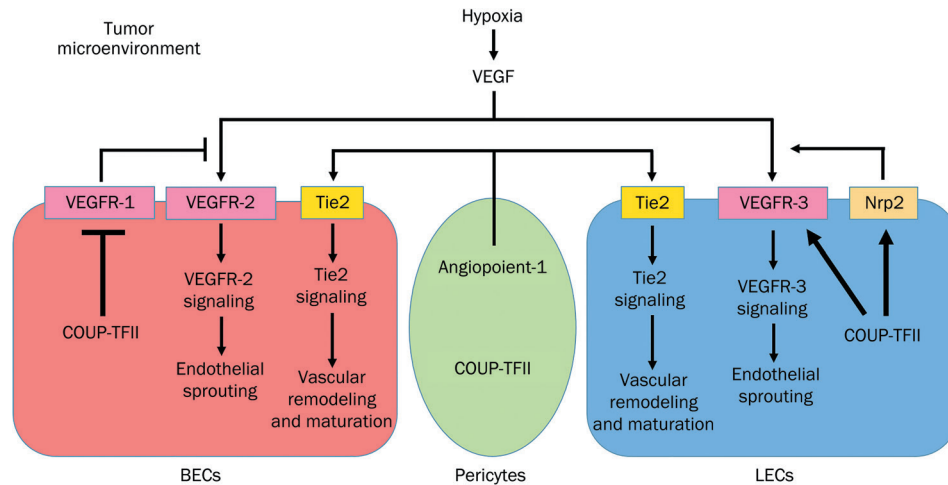
Figure 1. A model of COUP-TFII in the tumour microenvironment. In blood endothelial cells, COUP-TFII represses VEGFR-1 expression, a decoy receptor that sequesters the VEGF ligand, and leads to reduction of VEGFR-2 signalling. In contrast, in the lymphatic endothelial cells, COUP-TFII directly enhances the expression of VEGFR-3 and Nrp2, and promotes VEGFR-3 signalling. Additionally, COUP-TFII also upregulates the expression of Ang-1 in pericytes, and Ang-1 serves as a paracrine signal to activate Tie2 receptors on the endothelial cells. Thus, COUP-TFII regulates angiogenesis and lymphangiogenesis via the VEGF/VEGFR and Ang-1/Tie2 signalling pathways within the tumour microenvironment.

tate tumour, but not in the normal prostate counterparts. In *Pten*-null mice, hyperactive TGF-β signalling is repressed by COUP-TFII overexpression, while it is enhanced by COUP-TFII depletion[26], suggesting that COUP-TFII might override the TGF-β-induced growth barrier to promote prostate cancer progression. A mechanistic investigation revealed that COUP-TFII physically associates with SMAD4, and sequesters SMAD4 from binding to the promoter of target genes, therefore repressing TGF-β signalling[26]. Moreover, deletion of SMAD4 restored invasive tumour growth in *Pten*-null mice lacking COUP-TFII, demonstrating that COUP-TFII-mediated repression of TGF-β signalling is necessary for *Pten*-null prostate indolent tumours to develop into full-blown metastatic prostate cancers[26].

As shown in Figure 2, COUP-TFII plays a crucial role in inducing indolent prostate cancer to develop into metastatic-prone cancer by directly interacting with SMAD4 to destroy the TGF-β-induced growth barrier.

COUP-TFII in breast cancer
In addition to prostate cancer, increasing evidence suggests that COUP-TFII might play an important role in breast cancer progression. Using immunostaining, Nagasaki *et al* showed that 59% of 119 human breast cancer cases stained immunohistochemically positive for COUP-TFII[24]. Higher expression of COUP-TFII is associated with a worse prognosis and clinical outcome, as well as lymph node metastasis. Additionally, a positive correlation between COUP-TFII and ERα status was
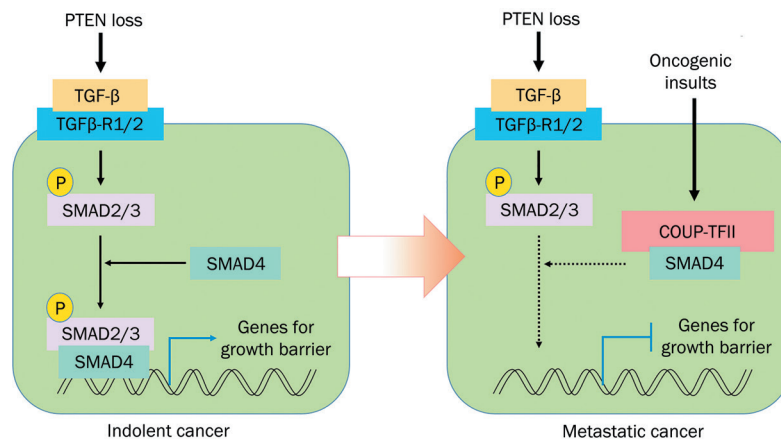

Figure 2. A model of COUP-TFII's role in prostate malignancy. PTEN inactivation drives prostate tumour initiation and progression. However, it also induces TGF-β signalling, which serves as a tumour growth barrier to restrict tumours in a premalignant state. In a later stage, alternative oncogenic insults stimulate COUP-TFII expression, which sequesters SMAD4 and blocks TGF-β signalling. Thus, COUP-TFII plays a crucial role in inducing indolent prostate cancers to develop into metastatic-prone cancers.

demonstrated[24, 30]. However, another report showed that a reduction in COUP-TFII associated with increased tumour stage in ERα-positive invasive ductal carcinomas[30], suggesting an inverse correlation between COUP-TFII and tumour grade. The contrasting results regarding COUP-TFII expression in different breast cancer samples might reflect its complicated roles during different breast cancer progression phases.

Interestingly, both reports showed a positive correlation between COUP-TFII and ERα status, consistent with previous studies, which revealed that siRNA knockdown of ERα in MCF-7 breast cancer cells decreased COUP-TFII expression, while treatment with estradiol increased the expression of COUP-TFII[31]. Reduction of COUP-TFII expression levels might render the breast cancer cells resistant to tamoxifen (TAM)[31]. Conversely, overexpression of COUP-TFII in TAM-resistant human breast cancer cell lines increases the effects of TAM and ICI 182780 treatment[31]. Additionally, COUP-TFII has been shown to interact with ERα and inhibit estrogen-induced gene expression[32], suggesting that COUP-TFII might mediate breast cancer cell sensitivity to TAM therapy through inhibition of the ERα activity.

The current results indicate that COUP-TFII might be involved in the initiation and progression of breast cancer, as well as in TAM resistance. Further research using appropriate animal models is needed to elucidate its precise function and the underlying mechanism, which might contribute to the future development of breast cancer treatment strategies.

COUP-TFII in colon and ovarian cancers

COUP-TFII immunostaining in 95 colorectal cancer patients indicated that COUP-TFII is positive in over 57% of the tumour samples, while there was a minimal expression of COUP-TFII in the normal colonic mucosa, and patients with COUP-TFII positive tumours had a better overall survival rate than those with COUP-TFII negative tumours[33]. In contrast, COUP-TFII is highly expressed in stromal and lowly expressed in epithelial cells of ovarian tumours, while this expression pattern is reversed in the normal ovarian tissue. The patients with high epithelial/stromal ratios of COUP-TFII expression exhibited a decrease in tumour recurrence[34]. Additional studies also showed a relatively low expression of COUP-TFII in ovarian tumours compared to non-tumour tissue[35]. These data suggested that COUP-TFII serves as both a tumour promoter and suppressor in different tumour types. While these analyses from tumour patients implied that COUP-TFII might be associated with colon and ovarian cancers, the precise role of COUP-TFII in these cancers remains to be investigated.

Summary and perspective

The studies reviewed here show that COUP-TFs, particularly COUP-TFII, are both up- and downregulated in different tumours and reveal a potential role for COUP-TFII in tumorigenesis. Although detailed analyses have illustrated the indispensable functions of COUP-TFII in prostate cancer and the tumour microenvironment, further investigations are needed to differentiate the distinct roles of COUP-TFII in the course of

different cancers' initiation and progression.

As shown above, COUP-TFII potently modulates multiple signalling pathways and controls both tumour cell growth and angiogenesis, which renders it an important target for anticancer intervention. We have already found that deletion of COUP-TFII in adult mice has no discernible phenotypes, suggesting that inhibiting COUP-TFII activity in the adult may have few toxic side effects. Moreover, COUP-TFII is a nuclear receptor, and its activity could potentially be regulated by ligands. Indeed, crystal structures of the COUP-TFII LBD domain illustrate that there are two small pockets for ligand binding and that retinoic acid may be a low-affinity ligand that activates COUP-TFII[36]. Thus, it would be highly feasible to find some small molecules that could bind COUP-TFII and regulate its activity. In future work, it will be important to screen for and identify specific antagonist ligands of COUP-TFII and examine their latent capacity for anticancer intervention.

Abbreviations

Ang-1, Angiopoietin-1; BECs, blood endothelial cells; CDH, congenital diaphragmatic hernia; COUP-TF, chicken ovalbumin upstream promoter transcription factor; CVI, cerebral visual impairment; DBD, DNA-binding domain; E10, embryonic day 10; ERα, estrogen receptor α; LBD, ligand-binding domain; LECs, lymphatic endothelial cells; Nrp2, neuropilin 2; TAM, tamoxifen; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor.

References

1   Sagami I, Tsai SY, Wang H, Tsai MJ, O'Malley BW. Identification of two factors required for transcription of the ovalbumin gene. Mol Cell Biol 1986; 6: 4259–67.

2   Wang LH, Tsai SY, Cook RG, Beattie WG, Tsai MJ, O'Malley BW. COUP transcription factor is a member of the steroid receptor superfamily. Nature 1989; 340: 163–6.

3   Pastorcic M, Wang H, Elbrecht A, Tsai SY, Tsai MJ, O'Malley BW. Control of transcription initiation *in vitro* requires binding of a transcription factor to the distal promoter of the ovalbumin gene. Mol Cell Biol 1986; 6: 2784–91.

4   Wang LH, Tsai SY, Sagami I, Tsai MJ, O'Malley BW. Purification and characterization of chicken ovalbumin upstream promoter transcription factor from HeLa cells. J Biol Chem 1987; 262: 16080–6.

5   Miyajima N, Kadowaki Y, Fukushige S, Shimizu S, Semba K, Yamanashi Y, *et al*. Identification of two novel members of erbA superfamily by molecular cloning: the gene products of the two are highly related to each other. Nucleic Acids Res 1988; 16: 11057–74.

6   Wang LH, Ing NH, Tsai SY, O'Malley BW, Tsai MJ. The COUP-TFs

compose a family of functionally related transcription factors. Gene Expr 1991; 1: 207–16.

7 Ladias JA, Karathanasis SK. Regulation of the apolipoprotein AI gene by ARP-1, a novel member of the steroid receptor superfamily. Science 1991; 251: 561–5.

8 Tsai SY, Tsai MJ. Chick ovalbumin upstream promoter-transcription factors (COUP-TFs): coming of age. Endocr Rev 1997; 18: 229–40.

9 Pereira FA, Tsai MJ, Tsai SY. COUP-TF orphan nuclear receptors in development and differentiation. Cell Mol Life Sci 2000; 57: 1388–98.

10 Bosch DG, Boonstra FN, Gonzaga-Jauregui C, Xu M, de Ligt J, Jhangiani S, *et al*. NR2F1 mutations cause optic atrophy with intellectual disability. Am J Hum Genet 2014; 94: 303–9.

11 Al Turki S, Manickaraj AK, Mercer CL, Gerety SS, Hitz MP, Lindsay S, *et al*. Rare variants in NR2F2 cause congenital heart defects in humans. Am J Hum Genet 2014; 94: 574–85.

12 Carmeliet P, Jain RK. Angiogenesis in cancer and other diseases. Nature 2000; 407: 249–57.

13 Pralhad T, Madhusudan S, Rajendrakumar K. Concept, mechanisms and therapeutics of angiogenesis in cancer and other diseases. J Pharm Pharmacol 2003; 55: 1045–53.

14 Pereira FA, Qiu Y, Zhou G, Tsai MJ, Tsai SY. The orphan nuclear receptor COUP-TFII is required for angiogenesis and heart development. Genes Dev 1999; 13: 1037–49.

15 Lin FJ, Chen X, Qin J, Hong YK, Tsai MJ, Tsai SY. Direct transcriptional regulation of neuropilin-2 by COUP-TFII modulates multiple steps in murine lymphatic vessel development. J Clin Invest 2010; 120: 1694–707.

16 Qin J, Chen X, Xie X, Tsai MJ, Tsai SY. COUP-TFII regulates tumor growth and metastasis by modulating tumor angiogenesis. Proc Natl Acad Sci U S A 2010; 107: 3687–92.

17 Qin J, Chen X, Yu-Lee LY, Tsai MJ, Tsai SY. Nuclear receptor COUP-TFII controls pancreatic islet tumor angiogenesis by regulating vascular endothelial growth factor/vascular endothelial growth factor receptor-2 signaling. Cancer Res 2010; 70: 8812–21.

18 Fukuhara S, Sako K, Noda K, Zhang J, Minami M, Mochizuki N. Angiopoietin-1/Tie2 receptor signaling in vascular quiescence and angiogenesis. Histol Histopathol 2010; 25: 387–96.

19 Fischer C, Mazzone M, Jonckx B, Carmeliet P. FLT1 and its ligands VEGFB and PlGF: drug targets for anti-angiogenic therapy? Nat Rev Cancer 2008; 8: 942–56.

20 You LR, Lin FJ, Lee CT, DeMayo FJ, Tsai MJ, Tsai SY. Suppression of Notch signalling by the COUP-TFII transcription factor regulates vein identity. Nature 2005; 435: 98–104.

21 Chen X, Qin J, Cheng CM, Tsai MJ, Tsai SY. COUP-TFII is a major regulator of cell cycle and Notch signaling pathways. Mol Endocrinol 2012; 26: 1268–77.

22 Engelmann D, Mayoli-Nussle D, Mayrhofer C, Furst K, Alla V, Stoll A, *et al*. E2F1 promotes angiogenesis through the VEGF-C/VEGFR-3 axis in a feedback loop for cooperative induction of PDGF-B. J Mol Cell Biol 2013; 5: 391–403.

23 Zhou W, Wang G, Guo S. Regulation of angiogenesis via Notch signaling in breast cancer and cancer stem cells. Biochim Biophys Acta 2013; 1836: 304–20.

24 Nagasaki S, Suzuki T, Miki Y, Akahira J, Shibata H, Ishida T, *et al*. Chicken ovalbumin upstream promoter transcription factor II in human breast carcinoma: possible regulator of lymphangiogenesis via vascular endothelial growth factor-C expression. Cancer Sci 2009; 100: 639–45.

25 Augustin HG, Koh GY, Thurston G, Alitalo K. Control of vascular morphogenesis and homeostasis through the angiopoietin-Tie system. Nat Rev Mol Cell Biol 2009; 10: 165–77.

26 Qin J, Wu SP, Creighton CJ, Dai F, Xie X, Cheng CM, *et al*. COUP-TFII inhibits TGF-beta-induced growth barrier to promote prostate tumorigenesis. Nature 2013; 493: 236–40.

27 Dhanasekaran SM, Barrette TR, Ghosh D, Shah R, Varambally S, Kurachi K, *et al*. Delineation of prognostic biomarkers in prostate cancer. Nature 2001; 412: 822–6.

28 Tomlins SA, Mehra R, Rhodes DR, Cao X, Wang L, Dhanasekaran SM, *et al*. Integrative molecular concept modeling of prostate cancer progression. Nat Genet 2007; 39: 41–51.

29 Ding Z, Wu CJ, Chu GC, Xiao Y, Ho D, Zhang J, *et al*. SMAD4-dependent barrier constrains prostate cancer growth and metastatic progression. Nature 2011; 470: 269–73.

30 Litchfield LM, Riggs KA, Hockenberry AM, Oliver LD, Barnhart KG, Cai J, *et al*. Identification and characterization of nucleolin as a COUP-TFII coactivator of retinoic acid receptor beta transcription in breast cancer cells. PLoS One 2012; 7: e38278.

31 Riggs KA, Wickramasinghe NS, Cochrum RK, Watts MB, Klinge CM. Decreased chicken ovalbumin upstream promoter transcription factor II expression in tamoxifen-resistant breast cancer cells. Cancer Res 2006; 66: 10188–98.

32 Klinge CM, Silver BF, Driscoll MD, Sathya G, Bambara RA, Hilf R. Chicken ovalbumin upstream promoter-transcription factor interacts with estrogen receptor, binds to estrogen response elements and half-sites, and inhibits estrogen-induced gene expression. J Biol Chem 1997; 272: 31465–74.

33 Shin SW, Kwon HC, Rho MS, Choi HJ, Kwak JY, Park JI. Clinical significance of chicken ovalbumin upstream promoter-transcription factor II expression in human colorectal cancer. Oncol Rep 2009; 21: 101–6.

34 Hawkins SM, Loomans HA, Wan YW, Ghosh-Choudhury T, Coffey D, Xiao W, *et al*. Expression and functional pathway analysis of nuclear receptor NR2F2 in ovarian cancer. J Clin Endocrinol Metab 2013; 98: E1152–62.

35 De Sousa Damiao R, Fujiyama Oshima CT, Stavale JN, Goncalves WJ. Analysis of the expression of estrogen receptor, progesterone receptor and chicken ovalbumin upstream promoter-transcription factor I in ovarian epithelial cancers and normal ovaries. Oncol Rep 2007; 18: 25–32.

36 Kruse SW, Suino-Powell K, Zhou XE, Kretschman JE, Reynolds R, Vonrhein C, *et al*. Identification of COUP-TFII orphan nuclear receptor as a retinoic acid-activated receptor. PLoS Biol 2008; 6: e227.