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Five endometrial cancer risk loci identified through genome-wide association analysis


* contributed equally to this work
* to whom correspondence should be addressed

1 Wellcome Trust Centre for Human Genetics, University of Oxford, Oxford, OX3 7BN, UK.
2 Centre for Cancer Genetic Epidemiology, Department of Public Health and Primary Care, University of Cambridge, Cambridge, CB1 8RN, UK.
3 Department of Genetics and Computational Biology, QIMR Berghofer Medical Research Institute, Brisbane, QLD, 4006, Australia.
4 Department of Clinical Genetics, St George’s, University of London, London, SW17 0RE, UK.
5 Hunter Medical Research Institute, John Hunter Hospital, Newcastle, NSW, 2305, Australia.
6 Centre for Clinical Epidemiology and Biostatistics, School of Medicine and Public Health, University of Newcastle, NSW, 2305, Australia.
7 Hunter Area Pathology Service, John Hunter Hospital, Newcastle, NSW, 2305, Australia.
8 Centre for Information Based Medicine, University of Newcastle, NSW, 2308, Australia.
9 School of Biomedical Sciences and Pharmacy, University of Newcastle, Newcastle, NSW, 2308, Australia.
10 Institute of Health and Biomedical Innovation, Queensland University of Technology, Brisbane, 4006, Australia.
11 Centre for Cancer Genetic Epidemiology, Department of Oncology, University of Cambridge, Cambridge, CB1 8RN, UK.
12 University of California at Los Angeles, Department of Medicine, Division of Hematology/Oncology, David Geffen School of Medicine, Los Angeles, CA, 90095, USA.
13 Department of Gynecology and Obstetrics, University Hospital Erlangen, Friedrich-Alexander University Erlangen-Nuremberg, Erlangen, 91054, Germany.
14 Institute of Human Genetics, University Hospital Erlangen, Friedrich-Alexander-University Erlangen-Nuremberg, Erlangen, 91054, Germany.
Department of Medical Epidemiology and Biostatistics, Karolinska Institutet, Stockholm, SE-171 77, Sweden.

Hannover Medical School, Gynaecology Research Unit, Hannover, 30625, Germany.

Department of Gynaecology, Jena University Hospital - Friedrich Schiller University, Jena, 07743, Germany.

Hannover Medical School, Clinics of Gynaecology and Obstetrics, Hannover, 30625, Germany.

Department of Obstetrics and Gynecology, Division of Gynecologic Oncology, University Hospitals, KU Leuven - University of Leuven, 3000, Belgium.

Vesalius Research Center, Leuven, 3000, Belgium.

Laboratory for Translational Genetics, Department of Oncology, University Hospitals Leuven, Leuven, 3000, Belgium.

Department of Obstetrics and Gynecology, Division of Gynecologic Oncology, Mayo Clinic, Rochester, MN, 55905, USA.

Department of Health Sciences Research, Mayo Clinic, Rochester, MN, 55905, USA.

Department of Biostatistics, University of Kansas Medical Center, Kansas City, KS, 66160, USA.

Centre for Cancerbiomarkers, Department of Clinical Science, The University of Bergen, 5020, Norway.

Department of Obstetrics and Gynecology, Haukeland University Hospital, Bergen, 5021, Norway.

School of Medicine and Public Health, University of Newcastle, Newcastle, NSW, 2308, Australia.

Department of Molecular Medicine and Surgery, Karolinska Institutet, Stockholm, SE-171 77, Sweden.

Department of Women’s and Children’s Health, Karolinska Institutet, Karolinska University Hospital, Stockholm, SE-171 77, Sweden.

Centre for Genomic Sciences, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Hong Kong SAR, China.

Colon Cancer Genetics Group, Institute of Genetics and Molecular Medicine, University of Edinburgh, Edinburgh, EH4 2XU, UK.

MRC Human Genetics Unit, Western General Hospital Edinburgh, Edinburgh, EH4 2XU, UK.

Division of Genetics and Epidemiology, Institute of Cancer Research, London, SM2 5NG, UK.

Centre for Epidemiology and Biostatistics, Melbourne School of Population and Global Health, The University of Melbourne, Vic, 3010, Australia.

Peter MacCallum Cancer Center, The University of Melbourne, Melbourne, 3002, Australia.

London School of Hygiene and Tropical Medicine, London, WC1E 7HT, UK.


Molecular Biology of Breast Cancer, Department of Gynecology and Obstetrics, University of Heidelberg, Heidelberg, 69120, Germany.

Molecular Epidemiology Group, German Cancer Research Center, DKFZ, Heidelberg, 69120, Germany.

Division of Clinical Epidemiology and Aging Research, German Cancer Research Center (DKFZ), Heidelberg, 69120, Germany.

Division of Preventive Oncology, German Cancer Research Center (DKFZ) and National Center for Tumor Diseases (NCT), Heidelberg, 69120, Germany

German Cancer Consortium (DKTK), German Cancer Research Center (DKFZ), Heidelberg, 69120, Germany

Department of Obstetrics and Gynecology, Division of Tumor Genetics, Technical University of Munich, Munich, 80333, Germany.

Dr. Margarete Fischer-Bosch-Institute of Clinical Pharmacology, Stuttgart, 70376, Germany.

University of Tübingen, Tübingen, 72074, Germany.

Division of Cancer Epidemiology, German Cancer Research Center (DKFZ), Heidelberg, 69120, Germany.

Department of Laboratory Medicine and Pathology, Mayo Clinic, Rochester, MN, 55905, USA.
48 Cancer Epidemiology Centre, Cancer Council Victoria, Melbourne, Vic, 3004, Australia.
49 Department of Epidemiology and Preventive Medicine, Monash University, Melbourne, Vic, 3004, Australia.
50 Department of Genetics, Institute for Cancer Research, The Norwegian Radium Hospital, Oslo, 0310, Norway.
51 The K.G. Jebsen Center for Breast Cancer Research, Institute for Clinical Medicine, Faculty of Medicine, University of Oslo, Oslo, 0316, Norway.
52 Department of Clinical Molecular Oncology, Division of Medicine, Akershus University Hospital, Lørenskog, 1478, Norway.
53 Sheffield Cancer Research, Department of Oncology, University of Sheffield, Sheffield, S10 2RX, UK.

Abbreviations: EC, endometrial cancer; CI, confidence interval; GWAS, genome-wide association study; LD, linkage disequilibrium; OR, odds ratio; kb, kilobase; Mb, megabase; PCA, principal components analysis; DHS, DNase1 hypersensitivity site.
Abstract

We conducted a meta-analysis of three endometrial cancer (EC) GWAS and two replication phases totaling 7,737 EC cases and 37,144 controls of European ancestry. Genome-wide imputation and meta-analysis identified five novel risk loci at genome-wide significance at likely regulatory regions on chromosomes 13q22.1 (rs11841589, near \textit{KLF5}), 6q22.31 (rs13328298, in \textit{LOC643623} and near \textit{HEY2} and \textit{NCOA7}), 8q24.21 (rs4733613, telomeric to \textit{MYC}), 15q15.1 (rs937213, in \textit{EIF2AK4}, near \textit{BMF}) and 14q32.33 (rs2498796, in \textit{AKT1} near \textit{SIVA1}). A second independent 8q24.21 signal (rs17232730) was found. Functional studies of the intergenic 13q22.1 locus showed that rs9600103 (pairwise $r^2=0.98$ with rs11841589) is located in a region of active chromatin that interacts with the \textit{KLF5} promoter region. The rs9600103-T EC protective allele suppressed gene expression \textit{in vitro} suggesting that the regulation of \textit{KLF5} expression, a gene linked to uterine development, is implicated in tumorigenesis. These findings provide enhanced insight into the genetic and biological basis of EC.
Endometrial cancer (EC) is the fourth most common cancer in women in the United States and Europe, and the most common cancer of the female reproductive system. The familial relative risk is ~2, but highly penetrant germline mutations in mismatch repair genes, and DNA polymerases account for only a small proportion of the familial aggregation. Our previous GWAS and subsequent fine-mapping identified the only two reported genome-wide significant EC risk loci, tagged by rs11263763 in HNF1B intron 1 and rs727479 in CYP19A1 intron 4.

To identify additional EC risk loci, we re-analysed data from our previous GWAS (ANECS, SEARCH datasets) and conducted a meta-analysis with two further studies (Supplementary Figure 1). The first was an independent GWAS; the National Study of Endometrial Cancer (NSECG), including 925 EC cases genotyped using the Illumina 660W array, 1,286 cancer-free controls from the CORGI/SP1 GWAS and 2,674 controls from the 1958 Birth Cohort. The second study comprised 4,330 EC cases and 26,849 controls from Europe, the United States and Australia, genotyped using a custom array designed by the Collaborative Oncological Gene-environment Study (COGS) initiative (Supplementary Table 1, Supplementary Note).

We first performed genome-wide imputation using 1000 Genomes Project data, allowing us to assess up to 8.6 million variants with allele frequency ≥1% across the different studies. Per-allele odds ratios and P-values for all SNPs in the GWAS and iCOGS were obtained using a logistic regression model. There was little evidence of systematic overdispersion of the test statistic (λGC=1.002-1.038, Supplementary Figure 3). A fixed-effects meta-analysis was conducted for all 2.3 million typed and well-imputed SNPs (info score>0.90) in a total of 6,542 EC cases and 36,393 controls. The strongest associations were with SNPs in LD with previously identified EC risk SNPs in HNF1B and CYP19A1 (Figure 1, Table 1). For fourteen 1.5Mb regions containing at least one novel SNP with Pmeta<10^-5, we performed regional imputation using an additional reference panel that comprised 196 high-coverage whole genome-sequenced UK individuals (Supplementary Table 2).

Five novel regions containing at least one EC risk SNP with Pmeta<10^-7 were identified and the most strongly associated SNP in each region was genotyped in an additional 1,195 NSECG EC cases and 751 controls using competitive allele-specific PCR (KASPar, KBiosciences) and the Fluidigm BioMark System (Supplementary Table 3). Duplicate samples displayed concordance >98.5% between different genotyping platforms (Supplementary Table 4). All five SNPs were associated with EC at genome-wide significance (P<5×10^-8, Table 1, Figure 2), and these associations remained highly
significant when analysis was restricted to cases with endometrioid subtype only. Endometrioid-only analysis did not reveal any additional risk loci. eQTL analysis (Online Methods) in normal uterine tissue, EC tumour and adjacent normal tissue did not yield any SNPs robustly associated with the expression of nearby genes at the EC risk loci (Supplementary Table 7). However, for each risk locus, bioinformatic analysis including cell-type-specific expression and histone modification data identified correlated SNPs within 500kb in likely enhancers and multiple potential regulatory targets (Supplementary Table 6, Supplementary Figure 5). The most compelling candidates for future functional analysis are described below.

rs13328298 (OR=1.13, 95%CI:1.09–1.18, P=3.73×10^{-10}) on 6q22.31 lies in the long non-coding RNA LOC643623, 54kb upstream of HEY2 and 86kb upstream of NCOA7. HEY2 is a helix-loop-helix transcriptional repressor in the Notch pathway, which maintains stem cells, and dysregulation has been associated with different cancers. NCOA7 modulates the activity of the estrogen receptor via direct binding.

The second locus (rs4733613, OR=0.84, 95%CI:0.80–0.89, P=3.09×10^{-9}) is at 8q24.21. Stepwise conditional logistic regression identified another independent signal in this region, rs17232730 (pairwise $r^2=0.02$, $P_{	ext{cond}}=1.29×10^{-5}$, Table 2). Both EC SNPs lie further from MYC (784-846kb telomeric) than most of the other cancer SNPs in the region, including those for cancers of the bladder, breast, colorectum, ovary and prostate. rs17232730 is in moderate LD with the ovarian cancer SNP rs10088218 ($r^2=0.43$), with both cancers sharing the same risk allele, but rs4733613 is not in LD ($r^2\leq0.02$) with any other cancer SNP in the region (Supplementary Figure 5). A role in tumorigenesis is implicated for several miRNAs in the region. Of these, miR-1207-5p is reported to repress TERT, a locus also implicated in EC risk.

The lead SNP at 15q15 (rs937213; OR=0.90, 95%CI:0.86–0.93, P=1.77×10^{-8}) lies within an intron of EIF2AK4. EIF2AK4 encodes a kinase that phosphorylates EIF2a and downregulates protein synthesis during cellular stress. Another nearby gene, BMF, encodes an apoptotic regulator moderately to highly expressed in glandular endometrial tissue.

At 14q42, the lead SNP rs2498796 (OR=0.89, 95%CI:0.85–0.93, P=3.55×10^{-8}) lies in intron 3 of oncogene AKT1, which is highly expressed in the endometrium. Several SNPs in LD with rs2498796 are bioinformatically linked with regulation of AKT1 and four other nearby genes (SIVA1, ZBTB42, ADSSL1 and INF2; Supplementary Table 6, Supplementary
**Figure 5**. *AKT1* acts in the PI3K/AKT/MTOR intracellular signaling pathway, which affects cell survival and proliferation\(^{35}\) and is activated in endometrial tumors\(^{36}\), especially aggressive disease\(^{37,38,39}\). *SIVA1* encodes an apoptosis regulatory protein that inhibits p53 activity\(^{40,41}\) and enhances epithelial–mesenchymal transition to promote motility and invasiveness of epithelial cells\(^{42}\). *INF2* expression is reported to act as a promigratory signal in gastric cancer cells treated with mycophenolic acid\(^{43}\).

The final novel EC SNP was rs11841589 (OR=1.15, 95%CI:1.11–1.21, P=4.83×10\(^{-11}\)) on chromosome 13q22.1, 163kb and 445kb downstream from Kruppel-like factors *KLF5* and *KLF12*, respectively. KLF5 is a transcription factor associated with cell cycle regulation, and it plays a role in uterine development, homoeostasis and tumorigenesis\(^{44–47}\). Elevated KLF5 levels are strongly correlated with activating *KRAS* mutations\(^{48}\) and KLF5 is targeted for degradation by the tumor suppressor FBXW7. Both *FBXW7* and *KRAS* are commonly mutated in EC\(^{49}\). rs11841589 was one of a group of five highly correlated SNPs (\(r^2\geq 0.98\)) surpassing genome wide significance in a 3kb LD block bounded by rs9600103 (P=8.70×10\(^{-11}\)) and rs11841589 (**Figure 4a**). There was no residual association signal at this locus (P\(_{\text{cond}}\) >0.05) after conditioning for rs11841589. Bioinformatic analysis suggested that the causal variant at the intergenic 13q22.1 locus may affect a regulatory element that modifies KLF5 expression (**Supplementary Figure 5**); rs9600103 overlaps a vertebrate conservation peak, and a DNaseI hypersensitivity site (DHS) in estrogen and tamoxifen-treated ENCODE\(^{50}\) Ishikawa cells (**Figure 4a**). In addition, in a Hi-C chromatin capture experiment in Hela S3 cells\(^{51}\), an interaction loop was observed between a segment containing the KLF5 promoter and the rs11841589/rs9600103 locus (P=0.004, **Supplementary Figure 6**).

We further investigated the epigenetic landscape of a 16kb region around rs11841589 and rs9600103 that contained the SNPs most strongly associated with EC, by analysis of three EC cell lines: Ishikawa is homozygous for the rs9600103-A and rs11841589-G high-risk alleles, and provided a comparison with the ENCODE data; ARK-2 is homozygous for the low-risk T alleles at both SNPs; and AN3CA is a non-*KLF5* expressing line that is homozygous for the high-risk alleles (**Supplementary Figure 7**). We conducted formaldehyde-assisted identification of regulatory elements (FAIRE, to identify regions of open chromatin), and chromatin immunoprecipitation (ChIP) using antibodies against H3K4Me2 (marker of transcription factor binding\(^{52}\)) and panH4Ac (marker of active chromatin). Although the anti-H4Ac ChIP did not display a consistent signal in the region, signals from FAIRE and anti-H3K4Me2 ChIP were specifically present in the KLF5-expressing lines and were co-located with the conservation peak and DHS from the
ENCODE data at rs9600103, providing strong evidence for open chromatin and transcription factor binding here (Figure 4a). We then conducted chromatin conformation capture experiments for the KLF5-expressing Ishikawa endometrial cancer cells and we found a significant interaction between the NcoI restriction fragment containing the rs11841589/rs9600103 risk loci SNPs and the promoter region of KLF5 (Figure 4b).

The regulatory nature of the region around rs9600103 and rs11841589 was investigated using allele-specific luciferase enhancer reporter assays in Ishikawa cells (Figure 4c). Paired t-tests were used to compare the relationships between fragments containing the rs11841589 and rs9600103 alleles, and the pGL3-Promoter reporter vector (no insert) control (Supplementary Table 8). Fragments containing the rs9600103-T, rs11841589-T and rs11841589-G alleles had activity significantly lower than that of the pGL3-Promoter control (P<0.014). In contrast, the construct containing the rs9600103-A risk allele had luciferase expression similar to the pGL3-Promoter control (P=0.23) and significantly higher than that of rs9600103-T (P=0.02), rs11841589-T (P=0.05) and rs11841589-G (P=0.04). These results suggest that the EC risk tagged by rs11841589 is at least partly due to a regulatory element containing rs9600103, which interacts with the KLF5 promoter region, and the risk rs9600103-A allele is likely associated with increased gene expression.

In summary, this meta-analysis identified five novel EC risk loci at genome-wide significance, bringing the total number of common EC risk loci identified by GWAS to seven (Figure 1). Together with other risk SNPs reaching study-wide significance, these explain ~1.6% of the EC familial relative risk. Novel EC risk SNPs lie in likely enhancers predicted to regulate genes or miRNAs with known or suspected roles in tumorigenesis, and we specifically showed that a functional SNP at 13q22.1 may sit within a transcriptional repressor of KLF5. Our findings further clarify the genetic etiology of EC, provide regions for functional follow-up, and add key information for future risk stratification models.

**Methods**

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturegenetics/.
Acknowledgments

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Table 1: Risk loci associated with EC at $P<5\times10^{-8}$ in the meta-analysis.

<table>
<thead>
<tr>
<th>Locus</th>
<th>SNP</th>
<th>Position</th>
<th>Nearby gene(s)</th>
<th>EA</th>
<th>OA</th>
<th>EAF</th>
<th>All histologies</th>
<th>P</th>
<th>$I^2$</th>
<th>Endometrioid histology</th>
<th>P</th>
<th>$I^2$</th>
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<td>13q22.1</td>
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<td>73,814,891</td>
<td>KLF5, KLF12</td>
<td>G</td>
<td>T</td>
<td>0.74</td>
<td>1.15 (1.11-1.21)</td>
<td>4.83×10^{-11}</td>
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<td>126,016,580</td>
<td>HEY2, NCOA7</td>
<td>G</td>
<td>A</td>
<td>0.58</td>
<td>1.13 (1.09-1.18)</td>
<td>3.73×10^{-10}</td>
<td>0.00</td>
<td>1.15 (1.11-1.20)</td>
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<td>8q24.21</td>
<td>rs4733613</td>
<td>129,599,278</td>
<td>MYC</td>
<td>G</td>
<td>C</td>
<td>0.87</td>
<td>0.84 (0.80-0.89)</td>
<td>3.09×10^{-9}</td>
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<td>EIF2AK, BMF</td>
<td>T</td>
<td>C</td>
<td>0.58</td>
<td>0.90 (0.86-0.93)</td>
<td>1.77×10^{-8}</td>
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<td>1.18 (1.13-1.23)</td>
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Positions in build 37; EA, Effect allele; OA, Other allele; EAF, effect allele frequency; $I^2$, heterogeneity $I^2$ statistic. For all novel loci, the lead SNP was either directly genotyped or imputed with an information score of more than 0.9. $HNF1B$ and $CYP19A1$ have been previously reported by Painter et al. and Thompson et al.
Table 2: Conditional analysis of 8q24 locus showing two independent association signals.

<table>
<thead>
<tr>
<th>SNP</th>
<th>Position</th>
<th>EA</th>
<th>OA</th>
<th>EAF</th>
<th>Pairwise $r^2$ with</th>
<th>All histology meta-analysis</th>
<th>Conditioning on rs4733613</th>
<th>Conditioning on rs17232730</th>
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</thead>
<tbody>
<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>rs4733613 rs17232730</td>
<td>Allelic OR (95%CI) $P$</td>
<td>Allelic OR (95%CI) $P$</td>
<td>Allelic OR (95%CI) $P$</td>
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<tr>
<td>rs4733613</td>
<td>129,599,278</td>
<td>G</td>
<td>C</td>
<td>0.87</td>
<td>0.02</td>
<td>0.84 (0.79-0.89) 5.64 × 10$^-9$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>rs17232730</td>
<td>129,537,746</td>
<td>G</td>
<td>C</td>
<td>0.88</td>
<td>0.02</td>
<td>1.17 (1.10-1.24) 4.46 × 10$^-7$</td>
<td>1.14 (1.08-1.22) 1.29 × 10$^-5$</td>
<td>-</td>
</tr>
<tr>
<td>rs10088218*</td>
<td>129,543,949</td>
<td>G</td>
<td>A</td>
<td>0.87</td>
<td>0.02 0.43</td>
<td>1.14 (1.07-1.20) 1.65 × 10$^-5$</td>
<td>1.12 (1.05-1.18) 2.92 × 10$^-4$</td>
<td>1.01 (0.91-1.12) 0.818</td>
</tr>
</tbody>
</table>

Positions in build 37; EA, Effect allele; OA, Other allele; EAF, effect allele frequency.

*rs10088218 is associated with ovarian cancer (all subtypes), with the association being more significant for cancers of serous histology. rs10088218-G is the risk allele for both EC and ovarian cancer.
References


Figure legends

Figure 1: EC meta-analysis Manhattan plot
Manhattan plot of $-\log_{10}$-transformed P-values from meta-analysis of 22 autosomes. There are seven loci surpassing genome wide significance including two known loci: 15q21 (CYP19A1) and 17q12 (HNF1B) and five novel loci: 6q22 (NCOA7, HEY2), 8q24 (MYC), 13q22 (KLF5), 14q32 (AKT1, SIVA1), 15q15 (EIF2AK4, BMF).

Figure 2: Forest plots of novel EC risk loci
The odds ratio and 95% confidence intervals of each study of the meta-analysis are listed and shown in the adjacent plot. The $I^2$ heterogeneity scores (all <0.4) suggest that there is no marked difference in effects between studies. The SNPs represented are: a) rs11841589 (13q22), b) rs13328298 (6q22), c) rs4733613 (8q24), d) rs17232730 (8q24, pairwise $r^2$ 0.02 with rs4733613), e) rs937213 (15q15) and f) rs2498796 (14q32).

Figure 3: Regional association plots for the five novel loci associated with EC.
The $-\log_{10}$ P-values from the meta-analysis and regional imputation for three GWAS and eight iCOGS groups are shown for SNPs at: a) 13q22.1, b) 6q22, c) & d) 8q24, e) 15q15 and f) 14q32.33. The SNP with the lowest P-value at each locus is labeled and marked as a purple diamond, and the dot color represents the LD with the top SNP. The blue line shows recombination rates in cM/Mb. All plotted SNPs are either genotyped or have an IMPUTE info score of more than 0.9 in all datasets. Supplementary Figure 4 displays similar regional association plots with a larger number of SNPs using a less stringent info score cut-off.

Figure 4: The 13q22.1 EC susceptibility locus
a) Diagram showing the 16kb region (position 73,804,930- 73,820,618) around rs11841589, rs9600103 and correlated SNPs rs7981863, rs7988505 and rs7989799 (black marks). FAIRE and ChIP assays with anti-H3K4Me2 and anti-H4Ac antibodies for three EC cell lines ARK-2 (rs9600103-TT), Ishikawa (rs9600103-AA) and AN3CA (rs9600103-AA) are shown, with the y-axis displaying enrichment normalized to non-crosslinked genomic DNA/sonicated input DNA, relative to the Rhodopsin promoter as a negative control using the ΔΔCt method. DNaseI hypersensitivity site (DHS) density signal in ENCODE EC Ishikawa cells (Supplementary Note) are shown, from experiments with cell lines treated with estrogen and tamoxifen. 100 vertebrates conservation is also displayed. Vertical dotted line represents the position of rs9600103.
b) 3C experiment for KLF5-expressing Ishikawa cells. Relative interaction frequencies between an Ncol restriction fragment containing risk SNPs rs9600103 and rs11841589 (bait fragment) with Ncol fragments across the region were calculated using qPCR with normalization to the signal from a control BAC 3C library and a non-interacting chromosomal region, using the ΔΔCt method. The graph shows the frequencies plotted against the fragment position on chromosome 13. A significant interaction is seen with the fragment containing a KLF5 transcriptional start site in Ishikawa cells.

c) Luciferase reporter assay to analyze the activity of 3kb fragments containing either rs9600103 or rs11841589 using the pGL3 promoter vector in Ishikawa cells. Green arrows represent the low-risk alleles, and red arrows the high-risk alleles. Error bars represent the standard error of the mean. Data were normalized by subtraction of background luminescence and normalized to pGL4 Renilla activity. Luciferase activity in the rs9600103-A risk allele was more than double than that of the rs9600103-T protective allele (P=0.018). Paired t-tests between the different fragments also showed that the rs9600103-A high-risk allele has significantly higher expression compared with both rs11841589 alleles (0.045, 0.039) (Supplementary Table 8). Schematic diagram displays position on chromosome 13 of the fragment sequences and the arrows represents the position of the two SNPs.