PAX8 and MECOM are interaction partners driving ovarian cancer

Melusine Bleu1,9, Fanny Mermet-Meillon1,9, Verena Apfel1,9, Louise Barys1,9, Laura Holzer1, Marianne Bachmann Salvy1, Rui Lopes1, Inès Amorim Monteiro Barbosa1, Cecile Delmas2, Alexandra Hinniger2, Suzanne Chau2, Markus Kaufmann2, Simon Haenni2, Karolin Berneiser2,8, Maria Wahle2, Ivana Moravec3, Alexandra Vissières3, Tania Poetsch3, Erik Ahrné3, Nathalie Carte3, Johannes Voeshol3, Elisabeth Bechter1, Jacques Hamon1, Marco Meyerhofer1, Dirk Erdmann1, Matteo Fischer1, Therese Stachyra1, Felix Freuler2, Sascha Gutmann2, César Fernández2, Tobias Schmelzle1, Ulrike Naumann2, Guglielmo Roma2, Kate Lawrenson4, Cristina Nieto-Oberhuber5, Amanda Cobos-Correa2, Stephane Ferretti1, Dirk Schübeler6,7 & Giorgio Giacomo Galli1

The transcription factor PAX8 is critical for the development of the thyroid and urogenital system. Comprehensive genomic screens furthermore indicate an additional oncogenic role for PAX8 in renal and ovarian cancers. While a plethora of PAX8-regulated genes in different contexts have been proposed, we still lack a mechanistic understanding of how PAX8 engages molecular complexes to drive disease-relevant oncogenic transcriptional programs. Here we show that protein isoforms originating from the MECOM locus form a complex with PAX8. These include MDS1-EVI1 (also called PRDM3) for which we map its interaction with PAX8 in vitro and in vivo. We show that PAX8 binds a large number of genomic sites and forms transcriptional hubs. At a subset of these, PAX8 together with PRDM3 regulates a specific gene expression module involved in adhesion and extracellular matrix. This gene module correlates with PAX8 and MECOM expression in large scale profiling of cell lines, patient-derived xenografts (PDXs) and clinical cases and stratifies gynecological cancer cases with worse prognosis. PRDM3 is amplified in ovarian cancers and we show that the MECOM locus and PAX8 sustain in vivo tumor growth, further supporting that the identified function of the MECOM locus underlies PAX8-driven oncogenic functions in ovarian cancer.

1 Disease area Oncology, Novartis Institutes for Biomedical Research, Basel, Switzerland. 2 Chemical Biology and Therapeutics, Novartis Institutes for Biomedical Research, Basel, Switzerland. 3 Analytical Sciences and Imaging, Novartis Institutes for Biomedical Research, Basel, Switzerland. 4 Cedars-Sinai Women’s Cancer Program at the Samuel Oschin Cancer Center, Los Angeles, CA, USA. 5 Global Discovery Chemistry, Novartis Institutes for Biomedical Research, Basel, Switzerland. 6 Friedrich Miescher Institute for Biomedical Research, University of Basel, Basel, Switzerland. 7 Faculty of Science, University of Basel, Basel, Switzerland. 8 Present address: Biozentrum, University of Basel, Basel, Switzerland. 9 These authors contributed equally: Melusine Bleu, Fanny Mermet-Meillon, Verena Apfel, Louise Barys. ✉ Email: giorgio.galli@novartis.com
Ovarian cancer is a heterogeneous disease accounting for >140,000 yearly deaths worldwide. Despite the development of new treatment paradigms, the improvement of overall survival of ovarian cancer patients over the past decade has been dismal, highlighting the need to identify new therapeutic targets, particularly for subtypes not linked to specific genetic aberrations, such as BRCA1/2 mutation.

Transcription factors (TFs) are key proteins governing lineage-specific gene expression programs. Epigenomic profiling revealed how a subset of TFs in each cell type engage highly active regulatory elements to drive the expression of genes important for physiological or pathological cell states. Large-scale functional genomics screens have identified critical TFs necessary for lineage-specific proliferation of cancer cells, and, in the case of ovarian cancer, indicated PAX8 as a key driver of cancer cell proliferation. PAX8 is mostly known as a developmentally TF required for the establishment of follicular thyroid cells in mice and humans; however, its role in cancer is still under investigation. We and others have previously reported cell cycle and metabolism gene expression programs controlled by PAX8 in the kidney or ovarian cancer cells by binding to enhancer elements. While a plethora of PAX8 target genes have been reported both in physiological and pathological contexts, a mechanistic understanding of how PAX8 exerts its oncogenic functions remains to be determined.

Here, we report the binary interaction between PAX8 and the products of the MECOM (MDS1–EVI1 complex locus) locus and dissect its function. MECOM is a transcriptional unit originally constituted by two main promoters (separated by 500 kb) driving the expression of the MDS1 and EVI1 proteins. However, a splicing event that occurs frequently in ovarian cancer and acute myeloid leukemia (AML) leads to the expression of the fusion protein MDS1–EVI1. This protein has been previously defined as PRDM3 due to the presence of a PR domain of histone methyltransferases. We demonstrate that the PAX8 DNA-binding domain engages a large number of genomic sites and, at a small subset of loci, recruits PRDM3 via its PR domain and an array of C2H2 zinc fingers. This complex regulates a defined gene expression module involved in cell adhesion and extracellular matrix formation. We demonstrate that both PAX8 and MECOM are critical TFs to sustain in vivo growth of ovarian tumors, likely by MECOM acting as a PAX8 cofactor mediating a subset of PAX8 oncogenic functions. Importantly, we define a PAX8–MECOM gene signature that characterizes patients of gynecological cancers with poor prognosis. Our molecular dissection analysis pinpointed a potential strategy to target the interaction of these oncogenic TFs.

**Results**

**PAX8 and MECOM reside in the same complex.** PAX8 is a TF involved in lineage specification of the thyroid and urogenital tract. Genetic screens point to PAX8 as a candidate oncogene for ovarian and kidney cancers by regulating a gene expression program controlling cell cycle and metabolic genes. While PAX8 has been shown to activate gene expression by recruiting acetyltransferases, an unbiased characterization of the TFs engaged by PAX8 to elicit its oncogenic program is lacking. To characterize PAX8 interaction network, we utilized the BioID system, in which the prokaryotic bacterial ligase BirA is fused to a gene of interest, allowing to label proximally engaged proteins. In order to study PAX8, we inserted a BioID-HA cassette into the endogenous PAX8 locus in IGROV-1 ovarian cancer cells using CRISPR-Cas9 (Supplementary Figure 1A). Fusion of the BioID tag with a T2A-mCherry cassette allowed enrichment by FACS sorting of positive integrants as evidenced by the emergence of the PAX8-BioID-HA fusion protein in bulk population analysis (Supplementary Figure 1B). Indeed, most of the derived clones from such enriched populations display expression of the PAX8-BioID-HA fusion at the expected molecular weight (Supplementary Figure 1C).

These cells were then used in a BioID experiment to identify the proximal interactome of PAX8 using streptavidin enrichment and quantitative mass spectrometry (MS) (Fig. 1A). Differential analysis revealed 106 proteins specifically enriched in cells labeled with biotin vs. control samples (Fig. 1A and Supplementary Data 1). Gene ontology analysis of the obtained hits demonstrates enrichment for proteins involved in transcription and DNA repair (Supplementary Figure 1D), compatible with the known nuclear roles for PAX8.

Further inspection of the list of hits revealed several histones as well as proteins involved in DNA damage and a diverse set of chromatin modifiers belonging to multiple complexes (Supplementary Figure 1E). Given the cell type-specific nature of PAX8 oncogenic phenotype, we focused on lineage-specific TFs enriched in our BioID-MS experiment. We were particularly intrigued by MECOM as this gene has been shown to be frequently amplified in ovarian cancer. The two best characterized proteins encoded by the MECOM locus are EVII and MDS1–EVI1 (hereafter called PRDM3), which differ by the presence of an N-terminal domain PR/SET domain (Supplementary Figure 1F). Western blot analyses from two different IGROV-1 PAX8-BioID clones readily validated the proximity engagement of two MECOM splice variants EVII and PRDM3 by PAX8 (Fig. 1C). This was further confirmed by endogenous co-immunoprecipitation experiments between MECOM variants and PAX8 in two cell lines (Fig. 1D). In addition, due to the enriched expression of PRDM3 in ovarian cancer cells, we validated that PRDM3 alone can interact with PAX8 by co-immunoprecipitation of ectopically expressed PAX8 and PRDM3 in HEK293 cells (Fig. 1E), as well as by complementation assay using a cellular NanoBit assay (Fig. 1F).

Collectively, our data indicate that PAX8 and MECOM splice variants, including the ovarian cancer-specific PRDM3, reside in the same protein complex.

**PAX8 DNA-binding domain engages PRDM3 in a binary interaction.** In order to dissect the molecular basis for the interaction between PAX8 and PRDM3, we generated a large set of PAX8 mutants using a mammalian in vitro transcription–translation (IVTT) system coupled to interaction analysis by NanoBit (Fig. 2A). PAX8 is composed of a N-terminal DNA-binding domain called Paired (PRD), a conserved octapeptide (OP), a truncated homeodomain (HD), and a C-terminal transactivation (TA) domain (Fig. 2A). Deletion of the PAX8 DNA-binding domain blunted the luciferase signal compared to deletion of PAX8 TA domain (Fig. 2B) while displaying similar protein expression levels (Supplementary Figure 2A). Importantly, point mutations abolishing PAX8 DNA-binding capacity do not significantly affect its interaction with PRDM3 (Fig. 2B), suggesting that structural elements within the DNA-binding domain are necessary for PRDM3 binding. In order to understand if the PAX8 DNA-binding domain was sufficient to interact with PRDM3, we probed each PAX8 domain in a minimal reconstituted IVTT system, again
coupled to NanoBit (Supplementary Figure 2B). Only the PAX8 PRD domain gave a strong interaction signal when mixed to PRDM3, confirming that the PAX8 DNA-binding domain is sufficient for the interaction (Fig. 2C). Similarly, in order to understand which domains of PRDM3 are involved in PAX8 interaction, we expressed partially overlapping protein fragments scanning the entire length of PRDM3 with the IVTT-NanoBit system (Fig. 2D). While none of the constructs of single domains of PRDM3 were sufficient to achieve maximal interaction, we identified a construct encompassing the PR/SET domain plus the first array of zinc fingers (ZnF 1–7) to display optimal binding to PAX8 DNA-binding domain (Fig. 2E).

To test our results with an orthologous in vitro method, we expressed and purified recombinant proteins encompassing PAX8 PRD domain (amino acid residues 9–135) and PRDM3 PR/SET and ZnF 1–4 domains (amino acid residues 2–345) (Supplementary Figure 2C). Two-dimensional nuclear magnetic resonance (NMR) spectroscopy observing 13C-15N-labeled PAX8 showed chemical shift perturbation for a subset of peaks upon addition of PRDM3 protein and substantial line broadening of all PAX8 resonances (Fig. 2F). In a complementary experiment where the methyl region of the proton spectra of PRDM3 was analyzed upon PAX8 addition, we observed peak broadening and changes in the resulting spectrum, significantly differing from the sum of the

TFs enriched in PAX8 BioID-MS

| Protein | Accession | pval  | Log2FC |
|---------|-----------|-------|--------|
| PAX8   | Q06710    | 0.002016 | 1.617565 |
| ZHX3   | Q9H4I2    | 0.00216  | 1.719364 |
| MECOM  | Q03112    | 0.01003  | 1.416971 |
| TFAP2A | P06549    | 0.002513 | 1.322084 |
| MEF2D  | Q14814    | 0.00713  | 1.310884 |
| ZNF281 | Q9Y299    | 0.008675 | 1.2671   |
| MGA    | Q8W19     | 0.000451 | 1.266668 |
| ZNF609 | O15014    | 0.001563 | 1.066837 |
| ZNF384 | Q8TF68    | 0.001536 | 1.056428 |
| ZH02   | Q9Y6X8    | 0.005429 | 1.003837 |

Fig. 1 PRDM3 interacts with PAX8. A BioID-MS results from IGROV-1-PAX8-BioID-T2A-mCherry cells. Blue dots represent proteins significantly enriched (P value < 0.01 and Log FC > 1). Red dot represents PAX8 and orange dot represents MECOM. B List of transcription factors enriched in PAX8-BioID IP-MS experiment. C Western blot from BioID-WB experiments in two different IGROV-1-PAX8-BioID-T2A-mCherry clones. The picture displays one representative image out of three independent experiments. D Endogenous co-immunoprecipitation between PAX8 and MECOM variants in MFE-319 and IGROV-1 cells. The picture displays one representative image out of three independent experiments. E Co-immunoprecipitation of ectopically expressed PAX8-HA and PRDM3 in HEK293 cells. The picture displays one representative image out of five independent experiments. F NanoBit assay in HEK293A cells transfected with PAX8-LgBit (Lg) and PRDM3-SmBit (Sm). HNF1B and PCBD1 are an unrelated pair used as positive and specificity controls. RLU relative luminescence unit. Data are presented as mean values ± SD from three biological replicates. Source data for Western blots and interaction measurements are provided as a Source Data file.
Together, this indicates interaction between the two proteins and argues for the formation of a binary complex independently of DNA presence. As a further test, we performed crosslinking MS between PAX8 and PRDM3. Treatment of a PAX8–PRDM3 complex with disuccinimidyl sulfoxide (DSSO) readily formed high-molecular-weight species observed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and matrix-assisted laser desorption ionization-time of flight MS (MALDI-MS) (Supplementary Figure S2E, S2F). Peptide mapping of these species revealed extensive intramolecular interactions between the PRDM3 PR domain and the neighboring ZnF array. In addition,
from both these regions, we observed intermolecular crosslinks converging onto the second half of the PAX8 PRD domain (Fig. 2G and Supplementary Figure S2G and Supplementary Data 2) further supporting the notion that both PR and ZnF 1–7 of PRDM3 are necessary to achieve optimal binding to PAX8. To validate our structural/interaction findings in cells, we performed a CRISPR-Tiling screening21 in ovarian and lung cancer cells. Sliding window analysis of the single guide RNAs (sgRNA) representation displayed stronger dropout for sgRNAs targeting PAX8 DNA-binding domain (particularly the second half), compared to the other domains (Fig. 2H). Importantly, the phenotype is specific to ovarian cells, while lung cancer cells are inert to PAX8 targeting (Fig. 2H). Collectively, our data demonstrate that PAX8 and PRDM3 engage in a direct binary interaction involving PAX8 DNA-binding domain and the N-terminal portion of PRDM3.

PAX8 recruits PRDM3 to chromatin. In order to identify the functional consequences of the PAX8–PRDM3 interaction on gene regulation, we performed PAX8 and PRDM3 chromatin immuno precipitation sequencing (ChIP-seq) (with an antibody raised against PRDM3 PR domain) in NIH-OVCA3 ovarian cancer cells. This revealed that PAX8 locates in over 30,000 genomic regions, while we detected ~7600 PRDM3 sites (Fig. 3A). Importantly, the majority of these PRDM3 sites (>60%) were also bound by PAX8, arguing that both factors colocalize on chromatin and further corroborating their tight relationship (Fig. 3A, Supplementary Figure S3A, and Supplementary Data 3). Both motif enrichment and de novo motif finding readily identified the PAX motif as significantly enriched in PAX8+ and PAX8+PRDM3+ sites (Fig. 3B). At the same time, we did not detect evidence of the reported MECOM motif, suggesting that PRDM3 engages chromatin via PAX8 binding.

Next, we asked if chromatin binding of both factors occurs in a dependent or independent fashion. Towards this goal, we performed ChIP-seq for each factor upon downregulation of the other. RNA interference (RNAi) knockdown was validated by ChIP-quantitative real-time PCR (ChIP-qPCR) on selected loci (Supplementary Figure 3B). Subsequent differential binding analyses revealed that, upon PAX8 knockdown, PRDM3 displayed a global loss of occupancy (Fig. 3C, D) despite marginal changes of total protein levels (Supplementary Figure 3C). On the contrary, MECOM knockdown did not significantly affect PAX8 occupancy genome-wide (Fig. 3C, D). These results document a role for PAX8 in recruiting PRDM3/MECOM as a cofactor at common genomic sites.

Next, we analyzed the nuclear distribution of PAX8 and PRDM3 by transient expression of fluorescently tagged proteins and confocal microscopy. PAX8-eGFP was unevenly distributed in the nucleus and forming hub-like structures resembling transcriptional condensates22 (Fig. 3E). In these hubs, ~40% of PAX8 molecules are not mobile as evidenced by fluorescent recovery after photobleaching (FRAP). In contrast, PRDM3 displayed a more homogeneous nuclear expression and, at PAX8 hubs, rapidly diffused after photobleaching (Fig. 3E). This suggests strong tethering of PAX8 protein molecules in hub-like structures and a more dynamic engagement of PRDM3, potentially as a PAX8 cofactor.

Next, we wanted to define the transcriptional impact elicited by PAX8–MECOM complex and we performed RNA sequencing (RNA-seq) upon silencing of either PAX8 or MECOM in a panel of five ovarian cancer cell lines, which display the highest sensitivity to both PAX8 and MECOM knockdown (see below). Transcriptomic analyses were performed 4 days following short hairpin RNA (shRNA) induction using a doxycycline-inducible hairpin against PAX89 and two independent hairpins against MECOM displaying similar target knockdown efficiency (Supplementary Figure 3D and Supplementary Data 4). In order to identify common target genes of PAX8–MECOM, we regressed out potential cell line–specific effects, which identified a set of 58 genes that displayed significant changes upon silencing of PAX8 or MECOM (Fig. 3F). Pathway enrichment analysis revealed that this PAX8–MECOM gene module was significantly enriched in genes functioning in the extracellular matrix, focal adhesions, and tumor growth factor-β signaling (Supplementary Figure 3E). Importantly, while either PAX8 or MECOM depletion regulated this gene set consistently, the effect was stronger when depleting PAX8 arguing that MECOM acts as a cofactor to modulate a subset of the PAX8 target genes. In order to ask if this effect can be recapitated in vivo and is not limited to cell lines in culture, we injected NIH:OVCA3-shPAX8/shMECOM cells (two independent hairpins each) in nude mice and treated mice for 1 week with either vehicle or doxycycline for transcriptomic profiling (Supplementary Figure 3F). Importantly, also in vivo, our identified gene set was modulated by both PAX8 and MECOM (Fig. 3F and Supplementary Data 4) and, again, shPAX8 perturbation inducing stronger transcriptional modulation, despite a milder knockdown efficiency (Fig. 3F and Supplementary Figure S3F). Collectively, our data suggest that PAX8 is a major TF in ovarian cancer cells by engaging a large number of genomic sites, while PRDM3 (MECOM) is specifically recruited by PAX8 at specific genomic loci to modulate a defined common gene module.

PAX8 and PRDM3 drive ovarian tumor growth. Large-scale functional genomic screens have classified PAX8 as an ovarian cancer dependency6. In light of our discovery of MECOM as an interactor of PAX8, we evaluated their relationship in such genetic screening datasets. We employed genome-wide RNAi or CRISPR datasets from DepMap (Dependency Map) and observed that, among ovarian models, the cell lines most sensitive to PAX8
knockdown/knockout (KO) are also the ones most sensitive to MECOM perturbations (Fig. 4A and Supplementary Figure S4A). We rigorously tested these findings by performing colony formation assays in multiple cell lines with independent genetic reagents (Supplementary Figure 4B). A characteristic feature of PAX8-sensitive lines is high expression levels of MECOM, which suggests the possibility that high MECOM expression could be a biomarker for PAX8 sensitivity (Fig. 4A and Supplementary Figure S4A).

Next, we asked if PAX8/MECOM dependency can be recapitulated in xenograft models in vivo. Using NIH-OVCAR3 cells bearing doxycycline-inducible shRNAs against PAX8 or...
MECOM confirmed that PAX8 silencing leads to profound regression (Fig. 4B), while MECOM loss induces tumor growth arrest/stasis (Fig. 4C). These different responses to PAX8 or MECOM knockdown is suggestive of a weaker contribution of MECOM to ovarian cancer growth, possibly due to its cofactor activity. Importantly, upon long-term and potent ablation of PAX8 in vivo, we observed a striking loss of MECOM proteins (Fig. 4D), while silencing of the latter left PAX8 levels unaffected (Supplementary Figure 4C). Such data are compatible with the role of PAX8 in recruiting PRDM3 to chromatin at a subset of common loci.

We then extended our findings to large-scale expression profiling of cell lines and patient-derived xenografts (PDXs). When models were ranked by the Signature score (PAX8–MECOM gene module derived from in vitro/in vivo RNA-seq in Fig. 3E), we observed a significant correlation with PAX8 and MECOM expression (Supplementary Figure 4D, E), extending the notion that PAX8 and MECOM control of a specific signature is consistent across a large set of models. In addition, by binning TCGA high-grade serous ovarian cancer (HGSOC) cases (n = 430) based on either PAX8 or PRDM3 expression quartiles, we observed that cases with high PAX8 and MECOM expression displayed significantly higher Signature Score compared to others (Fig. 4E). Survival analysis of ovarian and endometrial cancer patients displaying different levels of Signature Score revealed that cases displaying high Signature Scores (top quartile) exhibited significantly worse survival compared to patients with low scores (bottom quartile), suggesting that high PAX8/MECOM activity identifies a subset of patients bearing particularly aggressive tumors.

Discussion

We here report a detailed mechanistic characterization of the interaction between PAX8 and the MECOM gene product PRDM3. Using cellular, biochemical, and biophysical methods, we map this binary interaction to the PAX8 DNA-binding domain and PRDM3 PR domain and ZnF 1–7. We demonstrate that PAX8 binds a large number of genomic sites owing to its hub-like nuclear distribution, while PRDM3 gets recruited to a subset of PAX8 sites to drive a gene expression module involved in the extracellular matrix and cell adhesion. Importantly PAX8 and MECOM are necessary for ovarian tumor growth in vivo in the extracellular matrix and cell adhesion. Importantly, PAX8 subset of PAX8 sites to drive a gene expression module involved interaction between PAX8 and the PRDM3 domain and PRDM3 PR domain and ZnF 1

Fig. 3 PAX8 recruits PRDM3 to common binding regions. A Venn diagram showing the overlap of ChIP-seq peaks of PAX8 and PRDM3 in ovarian cancer cells. ***P < 0.001 represents the statistical significance of the overlap between PAX8 and PRDM3 using Fisher’s exact test. B (Top) Sequence logo representation of the top motif identified by de novo motif finding in PAX8°PRDM3° sites and alignment to known PAX8 motif. (Bottom) Motif enrichment analysis for known PAX8 motif in PAX8°PRDM3° ChIP-seq peaks. C UCSC genome browser snapshot of the MANSC1 locus showing ChIP-seq tracks of PAX8 and PRDM3 in ovarian cells following shRNA-mediated knockdown of PAX8 or MECOM. shCTRL is a negative control. D Differential binding analyses of PAX8 (left) and PRDM3 (right) upon MECOM or PAX8 knockdown, respectively. MA plot represents the distribution of Log FC (y-axis) and base mean coverage (x-axis). Dots represent peaks with statistically significant differences (numbers indicated). E Representative FRAP images of PAX8-eGFP (green) and mCherry-PRDM3 (magenta) signal in the nucleus of U2OS cell. Arrow points to the bleached region with PAX8 hub. Scale bar = 10 μm. Average FRAP curves and quantification were generated by EasyFRAP-web tool. Mobile fraction of PAX8 in bleached region = 0.6; half-recovery time T1/2 = 7 s; R2 = 1. Mobile fraction of PRDM3 in bleached region = 1; half-recovery time T1/2 = 15.2 s; R2 = 1. F Expression heatmap of 58 genes from gene modules identified from RNA-seq experiments in five ovarian cancer cell lines upon PAX8 or MECOM knockdown. Log FC for the same 58 genes from in vivo xenografts studies is also plotted. Source data for Western blots and qPCR are provided as a Source Data file.
PRDM3 has been reported as one of the driver oncogenes of AML,32,33 its role in solid tumors has never been explored in detail. We here report an additional role of PRDM3 in ovarian cancer as a PAX8 cofactor. In such a disease setting, the MECOM locus undergoes frequent amplification18 and PRDM3 expression (due to the locus alternative splicing event) is frequently occurring as evidenced by TCGA data analysis. Together with PAX8, PRDM3 regulates a gene expression module involved in the extracellular matrix and cell adhesion. Recent single-cell RNA-seq analyses of FTSEC revealed cellular heterogeneity characterized by the expression of specific signatures34. Importantly, among the identified gene modules, the one related to EMT, which is expressed from PAX8+ cells, once applied to the TCGA dataset, identifies a population of patients with poorer prognosis34. The fact that the genes positively regulated by PAX8 and MECOM also stratify patients with poorer prognosis suggests that indeed PAX8 cooperates with MECOM to regulate a gene expression program that promotes aggressive tumor phenotypes.

Combined, this study lays the foundation for studying PAX8 and MECOM as therapeutic targets for epithelial ovarian cancer. While attempts at identifying chemical matter inhibiting independently PAX2-5-8 or MECOM DNA-binding domains have been reported35,36, identification of ligands inhibiting their functions remains particularly challenging due to the disordered/ flexible nature of the proteins as well as the paucity of the

Fig. 4 PAX8 and MECOM sustain ovarian cancer growth. A Barplot showing sensitivity to PAX8 or MECOM KO as per CRISPR screens reported in DepMap portal. Bars are color coded by MECOM expression. B, C Tumor volume measurements of NIH:OVCAR3 cells bearing shRNAs against PAX8 (B) or MECOM (C). Trt start = day of starting daily doxycycline treatment. *P < 0.01 and ***P < 0.0001 signify significantly and highly significant differences to the respective vehicle (two-sided t test post hoc) on the last treatment day. Data are presented as mean ± SEM from n > 5 mice cohorts. D Western blot analysis of tumors from (B) 1 week after treatment start. E Boxplot of z-score expression of PAX8-MECOM Signature (Sig. Score) in TCGA ovarian cases (n = 608) binned according to MECOM (left) or PAX8 (right) expression quartiles. Boxplots represent median and first and third quartiles, and whiskers extend to 95th percentile. P values are based on two-sided Wilcoxon’s rank-sum test. F Kaplan–Meier curve of survival from TCGA ovarian and endometrial patients bearing high or low levels of Signature Score (top and bottom quartile, n = 746). P P value from log-rank test. Source data for Western blots and qPCRs are provided as a Source Data file.
structure/function data. Our report sheds light on a previously uncharacterized interaction surface between two oncogenic TFs, potentially guiding the rational design of novel therapeutics for the treatment of a population of ovarian cancer patients with a poor prognosis.

**Methods**

**Cell culture.** HEK293A and COV-318 were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Amimed) supplemented with 10% fetal bovine serum (FBS) (Seradigm), 1× l-glutamine (2 mM), 1× sodium pyruvate (1 mM), and 1× non-essential amino acid (0.1 mM). IGROV-1, Kuramochi, and Ovise were maintained in RPMI (Amimed) supplemented with 10% FBS, 1× l-glutamine, 1× sodium pyruvate, and 1× HEPES (10 mM). NIH3T3-C3AR were maintained in RPMI (Amimed) supplemented with 20% FBS, 1× l-glutamine, 1× sodium pyruvate, 1× HEPES, and 1× internal transcribed spacer. MFE-319 were cultured in RPMI1640 (1:1); 20% FBS, 1× l-glutamine, 1× sodium pyruvate, 1× HEPES. All cell lines were obtained from ATCC and tested for identity by single-nucleotide polymorphism genotyping and mycoplasma contamination. Doxycycline-inducible shRNA cell lines were generated by lentiviral transduction of pLKO-TET-ON constructs containing the following shRNA sequences: shPAX8_1581 5′-gagagt- cacacaaagattgc-3′; shMECOM_4482 5′-tgatacctatcagaaac-3′; and shME-COM_5198 5′-gcatgattcttgattaaaa-3′. IGROV-1_Cas9 was obtained by lentiviral transduction of a construct overexpressing spCas9 under EF1A promoter (name pVcX3-LV-c208). IGROV-1-Cas9-PAX8-BioID-HA-T2A-mCherry were generated by cotransfection of IGROV-1_Cas9 cells with the following sgRNAs (annealed in TracrRNA (IDT)) against exon 12 of PAX8 (PAX8_guide_1 5′-ctacagatggtcaaaggccg-3′ and PAX8_guide_2 5′-atgctgaaagctggtgca-3′) and a template repair encompassing 800 bp upstream and downstream of the cleavage site flanking an in-frame cassette encoding a second-generation biontin protein ligase (BioID2) fused to mCherry sequence. DharmFECT Duo Transfection Reagent was used. Positive signal cells were retrieved by FACS on a Sony Flow Cytometer model SH800S. Single-cell clones were then isolated and picked for further validation by Western blot.

**Gene expression analyses.** For Western blot analyses, cells were harvested and lysed in RIPA buffer supplemented with protease inhibitor cocktail (Roche). Protein samples were loaded on SDS-PAGE gels, transferred onto nitrocellulose membranes, and probed with the following antibodies: GAPDH (Cell Signaling, 8884; 1:1000 dilution), HA (BioLegend, 901501; 1:1000 dilution), Lbgt (R&D systems, MAB10026, 1:1000 dilution), MECOM (Cell Signaling, 2593; 1:1000 dilution), PAX8 (Cell Signaling, 5901P; 1:1000 dilution), PRDM3 (GenScript, U0869C; 1:100,000 dilution), VINCULIN (Sigma, V9311; 1:400 dilution), and HRP-anti-rabbit and HRP-anti-mouse (Cell Signaling).

**RNA isolation and cDNA synthesis.** Total RNA was isolated from cells using TRIzol reagent (Life Technologies) following the manufacturer’s instructions. DNA contamination was removed by treatment with DNase (Roche). The RNA was reverse transcribed to cDNA using the GoScript Reverse Transcription System (Promega). The cDNA was then amplified using qPCR primers. qPCR reactions were performed using the Fast SYBR Green master mix reagent (Life Technologies) on a StepOnePlus™ Real-Time PCR System (Applied Biosystems). The expression of GAPDH and β-actin was used as reference genes.

**Gene expression analyses.** The expression levels of the target genes were determined by qRT-PCR. qPCRs were performed using the Fast SYBR Green master mix reagent (Life Technologies) on a StepOnePlus™ Real-Time PCR System (Applied Biosystems). The expression of GAPDH and β-actin was used as reference genes.

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**Gene expression analyses.** The expression levels of the target genes were determined by qRT-PCR. qPCRs were performed using the Fast SYBR Green master mix reagent (Life Technologies) on a StepOnePlus™ Real-Time PCR System (Applied Biosystems). The expression of GAPDH and β-actin was used as reference genes.
Healthcare, UK) equilibrated with 20 mM HEPES, 150 mM NaCl, 0.5 mM TCEP, and 10% glycerol, pH 7.2. The fractions containing pure protein were pooled and concentrated using Amicon Ultracentrifugal filter units (Merck, Germany). The purity and concentration of the protein samples were determined by reverse-phase ultra-high-performance liquid chromatography (RP-UHPLC), measuring the absorbance at 210 nm. The concentration was calculated using a bovine serum albumin (BSA) standard curve as a reference. Identity and molecular weight of the PAX8(9–135)-LbBit protein was confirmed by LC-MS.

**PD3M (75–434)-SmBit purification.** Cell pellets were thawed and suspended in buffer A (50 mM Tris, 300 mM NaCl, 10% glycerol, pH 8) supplemented with complete protease inhibitor (Roche, Switzerland) and TurboNuclease (Merck, Germany). The cell lysate was cleared by centrifugation at 5 × 10^5 g for 30 min at 4 °C. The clarified cell lysate was loaded onto two 1 ml HisTALON columns (GE Healthcare, UK) mounted in series on an ÄKTA Pure chromatography system (GE Healthcare). Contaminating proteins were washed away with 10 column volumes of buffer A, and the His-tagged protein was eluted with a linear gradient of 0–500 mM imidazole. The purity and concentration of the protein samples were determined by RP-UHPLC, measuring the absorbance at 210 nm. The concentration was calculated using a BSA standard curve as a reference. Identity and molecular weight of the PD3M (75–434)-SmBit protein was confirmed by LC-MS.

**Mass spectrometry**

**BioID and quantitative MS.** For the BioID experiment, IGROV-1-Cas9-PAX8-BiD-T2A-mCherry cells were kept in (biotin-free) DMEM medium for 24 h to reduce endogenous biotinylation levels. Subsequently, cells were incubated with or without 10 μM biotin for 24 h and harvested for proteomic analysis. Cells pellet of biological triplicates for both conditions were lysed by sonication in RIPA lysis buffer (Millipore, 20–188 plus 0.1% SDS) and cleared by centrifugation for 20 min at 10,000 x g. Supernatants containing 5 μg of protein were incubated with 30 μl of streptavidin-agarose beads (Pierce High capacity Streptavidin-Agarose Resin) for 3 h at 4 °C. Beads were washed four times with RIPA buffer and transferred to 100 mM TEAB (triethylammonium bicarbonate) buffer (pH 8.5) containing 7 μg of a Trypsin/LysC mixture (Promega). On-bead digestion was carried out overnight at 37 °C. The samples were dried and analyzed by LC-MS-MS on a Q-Exactive HF-X mass spectrometer equipped with a 2 μm H+1-C1-triple quadrupole reactive probe with shielded x/y-gradient coils. The data were processed and analyzed with the software TopSpec 3.6 (Bruker, Switzerland).

**NGS-based technologies**

**RNA sequencing.** RNA was prepared from cells or tumors using RNeasy Mini Kit (Qiagen) and RNA-seq libraries were prepared using TruSeq RNA Library Prep Kit v2 (Illumina) according to the manufacturer’s recommendations. Libraries were sequenced on a HiSeq 2500 (Illumina). The raw reads were subjected to the standard pipeline for the Illumina HiSeq 2500 according to the manufacturer’s recommendations. Libraries were sequenced on a HiSeq 2500 (Illumina).

**ChIP-seq.** Cells were crosslinked in 1% formaldehyde in PBS for 10 min at room temperature, after which the reaction was stopped by the addition of 0.125 M glycine. Cells were lysed and harvested in ChIP buffer (100 mM Tris at pH 8.6, 0.3% SDS, 1% Triton X-100, and 5 mM EDTA) and the chromatin disrupted by sonication using a EpiSonic sonicator (Active Motif) to obtain fragments of average 200–500 bp in size. One hundred micrograms of chromatin was incubated with specific antibodies overnight. Antibodies used were PAX8 (Cell Signaling, 59019, 1:10), PRDM3 (GenScript, U0869CG110-1, 1.5μg). Immunoprecipitated complexes were recovered on Protein G Dynabeads (Invitrogen). DNA was eluted by reverse crosslinking (65 °C for 8 h in 1% SDS and 0.1 M NaHCO3 in TE buffer), and purified using SPRI Select beads (Reckem Coulter). Libraries for ChIP-seq were generated using Ovation® Ultralow Library System V2 (NuGEN) and barcodes were added using NEBNext Multiplex Oligos for Illumina (Index Primers Set 1) (NEB) according to the manufacturer’s recommendation.

**Confoiram imaging and FRAP.** U2OS cells were seeded on 8-well chambered ibidi coverslip (ibidi, #80826) at the density of 10,000 cells/well in DMEM + 10% FBS (no antibiotics). After 2 days of incubation, cells were transiently co-transfected with constructs expressing eGFP/mTurquoise2 fusions of PAX8 and mCherry/mVenus fusions of PRDM3 (125 ng:125 ng of DNAs/well) by Lipofectamine 3000 (Invitrogen, #L3000001) according to the manufacturer’s instructions using 0.4 mg of TMT labeling reagent per sample. After 24 h incubation, the six samples were processed with Proteome Discoverer (version 2.1) and in-house Python scripts for statistical analysis. Functional annotations were performed using DAVID (https://david.ncifcrf.gov/) using the Gene Ontology Biological Process All annotation.

**Crosslinking MS.** Crosslinking MS was carried out essentially as described before. Briefly, PAX8 (2–328, AviTag) and PRDM3 proteins were mixed in a 1:1 ratio (7 μM each, preincubated for 1 h at room temperature, and crosslinked with 1 or 2 mM DSSO (Thermo Scientific) for 1.5 h at room temperature and quenched with 20 mM NH4HCO3. The degree of covalent complex formation was evaluated by SDS-PAGE and MALDI-MS (Ultraflextreme II, Bruker), using the dried droplet method with a saturated ammonium solution in CHCN/HOAc (1:1) at a ratio of (75/25); v/v, with 0.1% TFA (v/v). MALDI-MS analyses were performed in linear mode using an external calibration with the protein calibration standard II (Bruker). For peptide level analysis, the crosslinked complex was processed using the PreOms iST Kit (PreOms) according to the manufacturer’s instructions. The final peptide samples were dried and resuspended in 0.1% formic acid in LC-MS grade water. Using a Luminos Fusion mass spectrometer, equipped with an Easy-nLC 1200 (Thermo Scientific), RP chromatography was performed on an Easyspray PepMap RSLC C18, 2 μm, 100 A, 75 μm × 15 cm column (Thermo Scientific). Crosslinked peptides were separated with a 180 min gradient from 2 to 80% of acetonitrile in 0.1% formic acid at a flow rate of 300 nl/min. MS/MS spectra were acquired using the CID-MS2-MS3 method. LC-MS/MS data were analyzed with the detailed Proteome Discoverer (versions 2.2–2.4, Thermo Scientific) scripts using the X!TANX node against a fasta database containing the two proteins of interest. Only crosslinked peptides with an X!TANX score >30 were reported. The protein–protein interaction mapping for the complex was visualized with the xNET viewer tool.

**Nuclear magnetic resonance.** Solutions of uniformly 13C,15N-labeled PAX8(9–135) and unlabeled PRDM3(2–345) were prepared at a concentration of 100 μM in NMR buffer (25 mM d5-Tris, 100 mM NaCl, 1 mM d5-DTSP, 10% D2O, pH 8.0). All NMR experiments were performed at protein concentrations of 50 μM, which were achieved either by carefully mixing buffered solutions at a 1:1 ratio for the complex or by diluting the individual proteins with the NMR buffer for the reference spectra. All samples contained 200 μM of 2,2-dimethyl-2-silapentane-5-sulfonate-d6 sodium salt, which was used as an internal standard. The NMR spectra were measured in 3 mm NMR tubes with a sample volume of 180 μl. For each sample, 1D ’H and 2D (1H,1H)-heteronuclear multiple quantum coherence spectra were recorded at 296 K on a Bruker Avance III HD 800 MHz NMR spectrometer equipped with a 5 mm 1H,1H-triple resonance cryogenic probe with shielded x/y-gradient coils. The data were processed and analyzed with the software TopSpin 3.6 (Bruker, Switzerland).

**Animal experimentation.** All animal experiments were performed according to procedures covered by permit number BS-1975 issued by the Cantonal Veterinary Office, Basel, Switzerland, and strictly adhered to the federal animal protection act and the federal animal protection code. All animals were permitted to adapt for 7 days and housed in a pathogen-controlled environment (five mice/type III cage) with access to food and water ad libitum and were identified with radio frequency identification (RFID) tagged transponders.

NIH-OCAR3 bearing different docycloclinducible shRNAs (10 million cells in Hank’s balanced salt solution:Matrigel 1:1) were subcutaneously injected in the
flank of 6-8-week-old female athymic nude mice (Charles River). When tumors reached a mean tumor volume of ~100–150 mm³, animals were randomized into different treatment groups based on similar tumor size (~6 ± 4 group). Tumor size was measured three times a week with a caliper. Tumor volume was calculated using the formula (length x width²) x n/6 and expressed in mm³. Data are presented as mean ± SEM. Differences between the changes in TVol were assessed on the endpoint using a t test post hoc. At completion of the experiment, mice were euthanized according to the protocol, tumors were isolated, snap frozen in liquid nitrogen, and pulverized for molecular analyses using Covaris CP20.

Bioinformatic analyses

RNA sequencing. Gene-level expression quantities were estimated by the Salmon algorithm. Differential expression analysis was performed with DESeq2. PAX8–PRDM3 gene signature was identified by selecting the median top and bottom 29 genes significantly modulated genes upon knockdown of PAX8 or MECom across all models tested in vitro. Raw data are currently being deposited to SRA.

ChiP-seq. ChiP-seq data were mapped to the human reference genome (hg19 assembly) using bowtie2. Duplicated reads were removed using the MarkDuplicates utility of the Picard tools (https://broadinstitute.github.io/picard) and peaks called using macs2 version 2.1.1 using a P value cutoff of -p 0.000000001. ENCODE blacklisted regions44 were dismissed and an IDR5 (irreproducible discovery rate) cutoff of 0.05 was applied on sample replicates. For global analyses of PAX8–PRDM3 binding, the union of PAX8 and PRDM3 peaks was used, whereas the overlap for co-occupied peaks was identified using DiffBind46 R package. The significance of overlaps between PAX8 and PRDM3 binding sites was assessed using a permutation test from R package ChiPeakAnno46 (peakPermTest, version 3.2.4).1 The consensus PAX8 peaks were centered on the cell line-specific peak summits. The ChiP-seq dataset was then plotted over a 5 kb window centered at summits and organized according to the three clusters. Heat maps were generated with the genomation R/Bioconductor package interface17. Motif finding was performed using Homer (http://homer.ucsd.edu/homer/motif/) with default parameters. Differential binding at the common PAX8–PRDM3 sites was performed on extracted binding read counts matrices using DESeq2. Raw data are currently being deposited to SRA.

Intersection with publicly available datasets. Normalized Z-scores for expression of the 58 genes PAX8–PRDM3 modules were calculated for CCLE, PTX, and TCGA (https://portal.gdc.cancer.gov/) datasets for samples belonging to ovarian lineage and compiled in a signature score (the sign of genes identified as inversely regulated by shPAX8/shMECOM were reversed). Correlations between gene-level expression quantities were estimated by the Salmon algorithm. Gene expression quantities were divided into the top or bottom quartile of expression of genes directly regulated by PAX8 across all models tested in vitro. Raw data are currently being deposited to SRA.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The Crosslinking MS data used in this study are available in the PRIDE database under accession code PXD021708. The BioID-M5 data generated in this study have been deposited in the PRIDE database under accession code PXD021709. ChiP-seq and RNA-seq data have been deposited in SRA under accession PRJNA655844 and PRJNA655836, respectively. The remaining data are available within the Article, Supplementary information, or available from the authors upon request. Source data are provided with this paper.

Code availability

Computational analyses have been performed using open source code as indicated in the “Methods” section. No proprietary code/software have been employed.

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Author contributions

M.B., V.A., F.M.-M., R.L., I.B., M.K., and A.C.-C. performed molecular and cellular biology experiments. I.B. and M.B.S. performed bioinformatic analyses. I.H. and S.F. performed and supervised the in vivo experiments. C.D., A.H., S.H., K.B., M.W., E.B., J.H., M.M., D.E., M.F., T.S., F.F., S.G., C.F., and C.N.-O. performed or supervised biochemical and biophysical measurements. I.M. performed imaging experiments. A.V., T.P., E.A., N.C., and J.V. performed or supervised mass spectrometry experiments. U.N. and G.R. supported NGS measurements. G.G.G. supervised the project and wrote the manuscript together with insights by T.S.c., K.L., and D.S.

Competing interests

All the authors affiliated with Novartis Institutes for Biomedical Research are employees of Novartis. The remaining authors declare no competing interests.

Additional information

Supplementary information

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Correspondence and requests for materials should be addressed to G.G.G.

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