

Original Research

Bias-Controlled Computational Prioritisation of Therapeutic Hypotheses From Heterogeneous Molecular Data Identifies PLK1 as the Leading MTX-Combination Candidate in Choriocarcinoma

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Academic Editor: Yudong Cai

Submitted: 2 July 2026 Revised: 17 August 2026 Accepted: 24 August 2026 Published: 30 September 2026

Abstract

Background: Methotrexate (MTX) resistance remains clinically relevant in gestational trophoblastic neoplasia and choriocarcinoma. Because disease-specific molecular cohorts are limited, computational prioritisation of therapeutic hypotheses from heterogeneous public data must explicitly distinguish experimental prioritisation from therapeutic validation. **Methods:** Protein and phosphoprotein changes from an MTX-resistant JEG-3 model were used as effect-size seeds for random walk with restart propagation across four molecular interaction networks. Computational robustness was assessed using dropout analyses, degree-preserving edge randomisation, and seed-degree-matched permutation. Orthogonal evidence from DepMap/PRISM, external transcriptomic datasets, DrugComb records, a normal-placenta single-cell reference, and normal-tissue RNA expression was integrated to evaluate biological plausibility and translational constraints. Candidate hypotheses were prioritised using transparent multi-criteria decision analysis (MCDA), and cross-modal agreement was summarised descriptively using rank concordance rather than a combined-probability test, because the evidence sources share overlapping cell-cycle and DNA-damage-repair signal and therefore do not satisfy the independence assumption required by such tests. **Results:** PLK1 ranked first across all 3000 MCDA perturbations, demonstrating stable prioritisation despite uncertainty in evidence weighting. Network propagation supported prioritisation but not discovery-level significance: the edge-degree-preserving empirical p value was 0.017 (BH-FDR, 0.479), and the seed-degree-matched p value was 0.048 (BH-FDR, 0.896). No candidate reached FDR significance in external transcriptomic meta-analysis or lineage- and proliferation-adjusted DepMap/PRISM regression. PLK1 was the only candidate ranked first across all four evaluated evidence analyses (both network null models, adjusted DepMap analysis, and external transcriptomic meta-analysis), indicating complete descriptive cross-modal rank concordance without assuming statistical independence across analyses. PLK1 also showed strong but nonselective basal dependency across three choriocarcinoma models. DrugComb contained one independent MTX plus PLK1-inhibitor observation with positive ZIP synergy, and the exploratory tumour-to-highest-critical-normal RNA ratio was 1.25. **Conclusions:** This bias-controlled computational prioritisation framework identifies MTX plus PLK1 inhibition as the highest-priority therapeutic hypothesis for experimental evaluation in choriocarcinoma. PARP1 and PRKDC remain secondary biomarker-stratified hypotheses. More broadly, the framework demonstrates how heterogeneous molecular data can be integrated into transparent, reproducible therapeutic hypothesis prioritisation while explicitly separating computational prioritisation from therapeutic validation.

Keywords: choriocarcinoma; gestational trophoblastic disease; methotrexate; drug resistance; neoplasm; Polo-like kinase 1; antineoplastic combined chemotherapy protocols; systems biology; computational biology; decision support techniques; network propagation; multi-criteria decision analysis

1. Introduction

Methotrexate (MTX) remains an important treatment component for low-risk gestational trophoblastic neoplasia (GTN) [1,2,3]. However, resistance to first-line single-agent MTX develops in a substantial proportion of patients, affecting approximately 25–30% of low-risk GTN cases in contemporary reviews [4]. This challenge is particularly pronounced in choriocarcinoma, a rare trophoblastic ma-

lignancy with limited disease-specific molecular datasets, matched clinical series, and MTX-combination evidence. After first-line MTX failure in low-risk GTN, second-line actinomycin D is commonly used, while multi-agent chemotherapy is generally reserved for patients who fail sequential single-agent chemotherapy [4], leaving molecularly guided MTX-combination strategies insufficiently supported for therapeutic validation. While this guideline-



based approach is clinically prudent and prioritises patient safety, it also limits opportunities to systematically prioritise new therapeutic targets or rational combination strategies from available molecular evidence.

The clinical problem extends beyond the occurrence of MTX resistance. Low-risk GTN remains highly curable even after first-line MTX failure, which represents a major clinical achievement but also limits the availability of large untreated or longitudinal disease-context cohorts for studying resistance biology. Second-line actinomycin D achieves sustained remission in roughly 72% of MTX-resistant low-risk cases [5] and in 75% in a further series [6]. Although only a minority of patients require an alternative therapeutic strategy, this subgroup remains clinically important because treatment escalation can increase toxicity, delay remission, and require multi-agent chemotherapy.

Mechanistically, MTX is a synthetic folic acid analogue that interferes with folate-dependent pathways required for nucleotide synthesis and DNA replication in rapidly proliferating cells [7]. MTX resistance in GTN and choriocarcinoma is biologically consistent with adaptive gene- and protein-level programs involving kinase signalling, oxidative-stress regulation, and broader proteomic remodelling [8,9,10,11]. However, the available molecular evidence is heterogeneous across assay platforms, model systems, and disease contexts, making candidate therapeutic hypotheses difficult to compare systematically. A target identified from a single omics layer may behave differently when evaluated as an MTX-combination strategy in the choriocarcinoma context. Consequently, robust computational prioritisation of heterogeneous molecular data is needed to distinguish consistently supported hypotheses from context-specific observations while explicitly accounting for uncertainty and potential analytical bias. Such an approach can rank experimentally testable combinations, identify evidence gaps, and define the experiments required to distinguish competing therapeutic hypotheses. Prior work has characterised individual layers of this adaptation. Wu et al. [8] showed that RSK2 activation upregulates SOX8 to promote MTX resistance in GTN; Jun et al. [9] applied quantitative proteomics to nominate novel regulators of choriocarcinoma MTX resistance; Georgiou et al. [10] combined proteomic and phosphoproteomic profiling of MTX-resistant JEG-3 cells and identified ATR- and CDK4/6-directed vulnerabilities; and Bolze et al. [11] identified HLA-G as a predictor of monochemotherapy resistance in gestational choriocarcinoma. Each provides a defensible single-modality signal, but none compares candidate hypotheses across modalities, quantifies how much of a signal reflects network topology or pan-essentiality, or separates computational prioritisation from therapeutic validation. That comparative step is the gap addressed here.

Accordingly, we framed this study as a computational prioritisation problem for experimental triage. Rather than asking whether a candidate is therapeutically val-

idated, we asked which MTX-combination hypothesis should be tested first, which alternatives should remain as secondary candidates, and which claims should be withheld until direct experimental evidence becomes available. To address these questions, we developed a bias-controlled computational prioritisation framework that integrates network propagation, pharmacogenomic evidence, drug-combination records, transcriptomic datasets, normal-placenta single-cell projections, and normal-tissue RNA counterscreens. Computational evidence was treated as structured support for experimentally testable hypotheses, while clinically relevant cautionary factors, including pan-essentiality and normal-tissue expression, were incorporated into transparent candidate prioritisation. The resulting framework provides both a reproducible strategy for integrating heterogeneous molecular data and a preclinical validation roadmap to guide rational MTX-combination testing in choriocarcinoma. Our primary objective was to identify the most defensible first experiment while explicitly defining its translational constraints.

2. Materials and Methods

2.1 Study Design

The study was designed as a bias-controlled public-data hypothesis-ranking analysis to prioritise MTX-combination hypotheses in choriocarcinoma. The analysis was anchored in the MTX-sensitive JEG-3 versus MTX-resistant JEG-3R proteomic and phosphoproteomic changes as hypothesis-generating seed evidence reported by Georgiou et al. [10], which had identified DNA-damage repair and cell-cycle regulation as major programs associated with MTX-resistant choriocarcinoma. Terms such as synthetic lethality, validation, and therapeutic efficacy were reserved for conclusions that would require direct experimental evidence.

Protein and phosphoprotein effect-size signals from the MTX-sensitive JEG-3 versus MTX-resistant JEG-3R comparison were first used as disease-context seeds and propagated through public interaction networks. The resulting candidates were assessed with network-level bias controls and global null models to reduce the influence of graph topology, seed placement, and network-degree effects. A candidate target panel was then defined for structured downstream analysis. Transparent MCDA and uncertainty analysis were applied to rank these targets and identify the lead hypothesis. In parallel, orthogonal context and clinical risk layers examined biological plausibility and translational constraints, including transcriptomic support, pharmacogenomic and drug-combination evidence, trophoblast-state context, and normal-tissue expression. The overall workflow is summarised in Fig. 1.

Signals replicated in non-trophoblastic MTX-resistance datasets were considered external context, not disease-specific validation. DepMap/PRISM analyses were considered pan-cancer stress tests because they provide

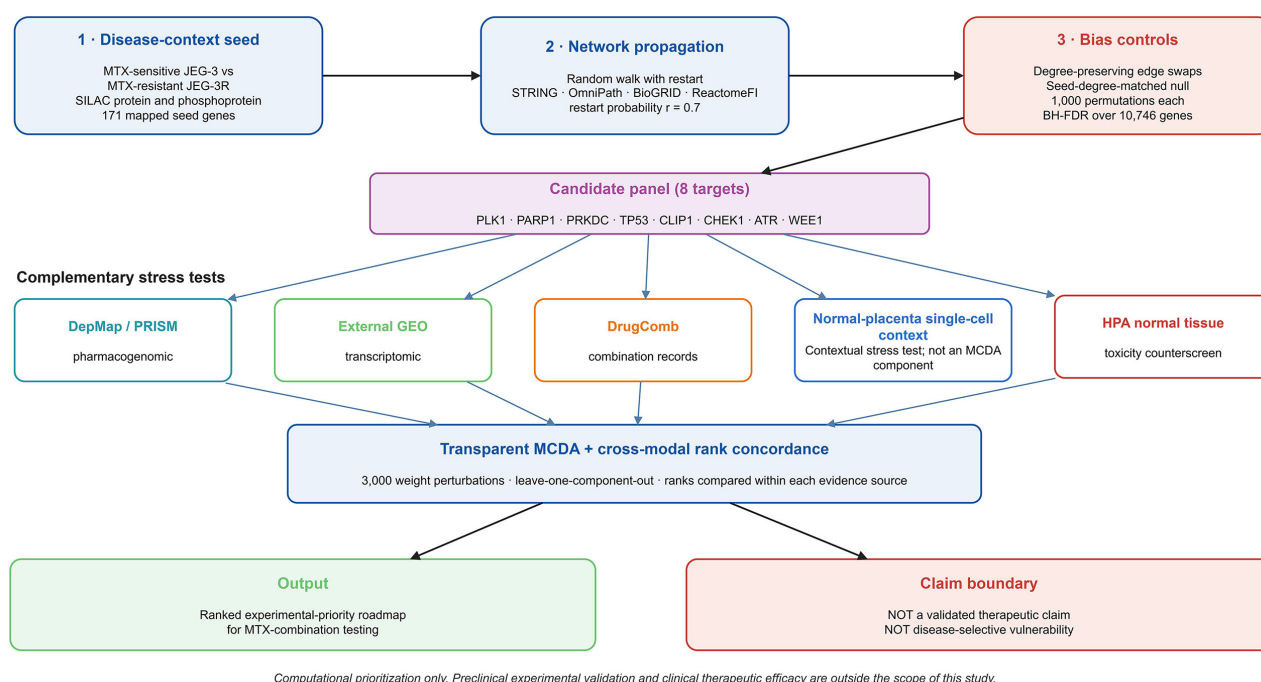


Fig. 1. Bias-controlled public-data workflow and claim boundary. Protein and phosphoprotein effect-size seeds from the Methotrexate (MTX)-sensitive versus MTX-resistant JEG-3/JEG-3R comparison were propagated through public interaction networks and then stress-tested with global network nulls, DepMap/PRISM, external transcriptomic datasets, DrugComb, normal-placenta single-cell data and normal-tissue RNA expression. The final output is an experimental-priority roadmap for MTX-combination testing, not a validated therapeutic claim.

scale but lack matched choriocarcinoma MTX-sensitivity measurements. DrugComb evidence was treated as direct combination context only when MTX itself was present and when records could be collapsed to independent drug-pair and cell-line units. Normal-tissue RNA comparisons were interpreted as toxicity-awareness heuristics and were not used as pharmacologic therapeutic-index estimates.

Candidate selection was deliberately conservative. The final evaluated panel included PLK1, PARP1, PRKDC, TP53, CLIP1, CHEK1, ATR and WEE1 because these targets represented recurrent network, DNA-repair, mitotic-checkpoint and translationally actionable hypotheses emerging from the public-data workflow. Tier labels from earlier exploratory analyses were not used as model inputs. This avoided circularity in which a manually assigned priority label would be rediscovered by a downstream scoring procedure.

No new patient samples, cell-line experiments, or unpublished clinical measurements were generated. This computational study was designed to provide scientific value by guiding the selection of combination experiments for trophoblastic models, not claiming therapeutic efficacy.

2.2 Data Sources and Evidence Layers

2.2.1 Proteomic and Phosphoproteomic Seed Data

Protein and phosphoprotein abundance changes from a stable isotope labelling by amino acids in cell culture (SILAC) quantitative mass spectrometry comparison of MTX-sensitive JEG-3 and MTX-resistant JEG-3R choriocarcinoma cells were obtained from Georgiou et al. [10] (Imperial College London, London, UK). This originating comparison does not include independent biological replicates and therefore carries unquantified measurement error. It was consequently treated as hypothesis-generating seed evidence only, and no inferential claim in this study is derived from the seed data themselves. Descriptive protein-phosphoprotein effect-size concordance in the originating SILAC dataset is shown in **Supplementary Fig. 1**. This constraint is the explicit reason the analysis was constructed as a bias-controlled prioritisation framework: seed reliability is stress-tested downstream by seed-dropout analysis, by separate propagation of the protein and phosphoprotein layers, and by a seed-degree-matched null model.

2.2.2 Public Interaction Networks and Bias Controls

Seeds were propagated across four public protein interaction resources: STRING v12.0 (University of Zurich, Zurich, Switzerland), OmniPath (European Molecular Biology Laboratory, Heidelberg, Germany), BioGRID release

4.4 (University of Toronto, Toronto, Canada), and ReactomeFI (Ontario Institute for Cancer Research, Toronto, Canada).

Two global null models were applied using the STRING graph as the reference network. Benjamini-Hochberg false discovery rate (FDR) correction was applied across the evaluated gene universe.

2.2.3 Transcriptomic and Pharmacogenomic Data

Gene expression data from five non-trophoblastic MTX-resistance datasets (GSE16066, GSE16070, GSE16080, GSE16082, GSE16089) and one disease-context trophoblastic dataset (GSE88873) were retrieved from the Gene Expression Omnibus (GEO; National Center for Biotechnology Information, Bethesda, MD, USA). A perturbation-context dataset (GSE192446) including vorinostat versus dimethyl sulfoxide transcriptional profiles in JEG-3 and JAR cells was also obtained from GEO and used as a phenotypic reversal reference.

CRISPR-Cas9 genome-scale gene effect scores were obtained from DepMap Public 26Q1 (Broad Institute, Cambridge, MA, USA). Drug sensitivity data for MTX were obtained from the PRISM Repurposing dataset, accessed through the DepMap portal. Three choriocarcinoma cell lines available in DepMap were included for disease-context dependency analysis: JAR (HTB-144), JEG-3 (HTB-36), both available from the American Type Culture Collection (ATCC, Manassas, VA, USA), and T3M-3 (RCB1018; RRID:CVCL_8065), catalogued by the RIKEN BioResource Research Center Cell Bank (Tsukuba, Japan). Pathway analysis used preranked gene set enrichment analysis (GSEA) applied to Hallmark gene sets from the Molecular Signatures Database (MSigDB; Broad Institute, Cambridge, MA, USA) and Reactome gene set collections (Ontario Institute for Cancer Research, Toronto, Canada).

Drug combination synergy records were obtained from DrugComb (Finnish Institute for Molecular Medicine, Helsinki, Finland), and from the Therapeutics Data Commons (TDC) DrugComb DrugSyn resource (Harvard Medical School, Boston, MA, USA). Single-cell trophoblast context was assessed using the GSE89497 normal-placenta dataset from GEO and the Vento-Tormo first-trimester atlas (E-MTAB-6701) from ArrayExpress (European Bioinformatics Institute, Hinxton, UK).

2.2.4 Normal-Tissue RNA Reference Data

Normal-tissue RNA consensus expression values were obtained from the Human Protein Atlas (KTH Royal Institute of Technology, Stockholm, Sweden), reported in normalised transcripts per million (nTPM). These data were used as the reference source for the normal-tissue expression counterscreen.

2.3 Network Propagation and Bias Controls

Weighted omics seeds were propagated across STRING, OmniPath, BioGRID, and ReactomeFI networks [12,13,14,15] using Random Walk with Restart [16]. In this algorithm, a virtual walker departs from seed nodes and at each step either moves to a neighbouring node with probability $(1 - r)$ or returns to the seed distribution with restart probability r ($r = 0.7$). Iteration continues until the node-score vector converges. Nodes receiving consistently high stationary scores across iterations were considered candidates supported by network proximity to the seed set. A total of 171 proteins and phosphoproteins meeting the published significance threshold were successfully mapped to gene identifiers and used as seed nodes.

As propagation scores in protein interaction networks can be inflated by hub node connectivity rather than biological relevance to the phenotype of interest, bias controls were applied in the form of two complementary global null models on the STRING graph. First, degree-preserving double-edge swaps were used to randomise network wiring across 1000 permutations, testing whether candidate scores were exceptional given the network's degree structure. Second, the STRING graph was held fixed while 171 mapped seeds were replaced by genes from matched degree bins, testing whether the target markers reflected the biological signal of the seed proteins or just a consequence of their favourable position in the network. A candidate could be nominally supported by one null and not the other; therefore, both were reported and interpreted jointly. Benjamini-Hochberg FDR correction [17] was applied across the full evaluated universe of 10,746 genes to account for multiple testing, and candidates that did not survive this correction were not considered sufficient to support discovery-level network claims, even when nominal p -values were below 0.05.

Candidate robustness was further evaluated by three dropout analyses to test whether rankings were stable across data sources. Network leave-one-out tested whether a candidate ranking depends on any single network source. Seed-dropout analysis tested whether ranking persisted when portions of the omics seed set were removed. Omics-layer-dropout repeated network propagation using protein and phosphoprotein seed signals separately, allowing us to assess whether candidate support was robust across measurement layers or dominated by one omics modality. These analyses were not designed to create additional p -values; they were used to distinguish broadly supported candidates from artefacts of a single data source.

2.4 Orthogonal Stress Tests

2.4.1 Pharmacogenomic and Transcriptomic Analysis

After a disease-context candidate panel was defined from network propagation and bias controls, orthogonal public-data analyses were performed to evaluate whether these candidates retained biological and pharma-

cogenomic plausibility across independent resources. Accordingly, DepMap CRISPR gene-effect data and PRISM MTX-sensitivity profiles were used as pharmacogenomic stress tests [18,19,20]. DepMap assessed basal dependency of candidate targets, including in JAR, JEG-3, and T3M-3 choriocarcinoma models, whereas PRISM evaluated candidate-associated patterns in available MTX drug-sensitivity data. These analyses were interpreted cautiously because broad gene dependency may indicate experimental tractability, but it does not necessarily imply MTX-specific sensitisation or clinical exploitability. For this reason, DepMap CRISPR results were summarised separately from pan-cancer regression models and were interpreted as basal dependency evidence only. PRISM MTX data were not available for the three public choriocarcinoma models, so no disease-specific public drug response validation was claimed.

External transcriptomic stress tests were evaluated using MTX-resistance datasets from GEO [21]. Gene expression data from five non-trophoblastic MTX-resistance datasets (GSE16066, GSE16070, GSE16080, GSE16082, GSE16089) were pooled using random-effects meta-analysis [22]. This approach was chosen to accommodate heterogeneity in tissue origin, assay platform, and experimental design, rather than to force a disease-specific pooled estimate. The choriocarcinoma trophoblastic dataset (GSE88873) was retained but reported descriptively only, as its MTX-sensitive arm contained a single sample and was therefore unsuitable for inferential pooling. Preranked GSEA [23] was used to examine pathway-level patterns across Hallmark and Reactome collections, in which genes were ranked by log₂ fold change from each dataset to identify biological processes consistently enriched across MTX-resistance contexts. Mechanism-keyword prefiltering was deliberately omitted to avoid introducing prior bias toward expected pathways.

2.4.2 Single-Cell Contextualisation

Single-cell analysis was used to contextualise the MTX-resistance signature within trophoblast lineage states. Given the trophoblastic origin of choriocarcinoma, this analysis examined whether the JEG-3R resistance signature preferentially aligned with specific normal trophoblast compartments, thereby providing lineage-state context for interpreting MTX-resistance biology.

The top 100 upregulated and top 100 downregulated GSE88873 JEG-3R genes were projected onto the GSE89497 normal-placenta single-cell expression matrix from the human placental single-cell RNA-seq reference reported by Liu et al. [24]. An AddModuleScore-like procedure was applied to log₁p(TPM) values, in which target gene scores were corrected against expression-bin-matched background genes to account for differences in mean expression level across cells. The resistance score was defined as the upregulated-module score minus the downregulated-

module score. Cell-level findings were reported descriptively because many cells may originate from a limited number of biological samples, making cell-level *p*-values unsuitable for patient-level inference.

As a predefined-panel sensitivity analysis, the final genes were projected onto both GSE89497 and the larger Vento-Tormo E-MTAB-6701 normal first-trimester atlas [25]. Because several candidate genes are involved in cell cycle regulation and mitotic progression, a separate proliferation score was computed using established proliferation marker genes (MKI67, TOP2A, PCNA, MCM2, MCM5, CCNB1, CDK1, and AURKB) with no overlap with the target panel, to distinguish candidate-specific expression patterns from general proliferative activity. E-MTAB-6701 cells were pseudobulked by individual and trophoblast subtype before paired comparisons. A small-sample sensitivity model used individual fixed effects and individual-clustered standard errors to estimate subtype differences after adjustment for the proliferation score.

2.4.3 Drug-Combination Evidence

Drug combination synergy records from DrugComb were used to identify whether any direct MTX and candidate inhibitor combination evidence existed in the public domain [26]. MTX combinations were separated from other antifolates. Before analysis, records were collapsed into independent units defined by antifolate type, target class, drug pair, and cell line to avoid overcounting repeated doses, replicate matrices, or closely related records that could overstate evidence density. MTX was separated from pralatrexate and other antifolates because antifolates can differ in cellular transport, polyglutamation, target engagement, or resistance biology. Given the sparse number of independent records after collapse, positive results were reported descriptively only.

As a separate exploratory prioritisation layer, the public TDC DrugComb DrugSyn file was used to fit ExtraTrees regression models for ZIP and Bliss scores [27,28,29]. Symmetric Morgan fingerprint features were generated for each drug pair so that the order of the drugs did not affect the model input. These chemical features were evaluated alone and together with a 16-component RNA embedding learned from the 59 NCI-60 cell lines represented in the TDC DrugComb DrugSyn training data. JEG-3 DepMap RNA values were gene-matched and projected into that embedding. Model generalisability was assessed using three data-splitting strategies that followed the TDC data-splitting framework and common drug-synergy prediction evaluation settings: random-row, cold drug-pair, and cold cell-line splits. Because JEG-3 is outside the NCI-60 training domain and the proposed inhibitors were not exact training compounds, these estimates were treated as in-silico assay-ordering priors only, not calibrated JEG-3 synergy predictions.

2.5 Clinical Risk Assessment

Clinical risk assessment was performed as a normal-tissue RNA counterscreen for the candidate target panel. RNA expression values from the three DepMap choriocarcinoma models were back-transformed where applicable and compared descriptively with the Human Protein Atlas [30] consensus RNA expression values in selected critical normal tissues. The selected tissues included proliferative or treatment-relevant compartments, such as bone marrow, thymus, lymphoid tissue, and other normal tissues considered relevant for toxicity-aware preclinical planning. Because these two sources use different measurement platforms, the resulting ratios were treated as expression window estimates rather than precise quantitative comparisons.

Normal-tissue RNA expression was used only as a warning layer. A high tumour-to-normal RNA ratio does not ensure efficacy, and a low ratio does not prove toxicity. The intent was to prioritise experiments with toxicity awareness, not to estimate patient therapeutic index.

2.6 Candidate Ranking and Exploratory Cross-Modal Integration

Transparent MCDA was used to integrate supportive evidence and translational caution signals across the public data workflow [31]. Transparent MCDA was used because the evidence layers were heterogeneous and could not be reduced to a single validated likelihood model. Higher scores reflected stronger biological and pharmacogenomic support, while pan-essentiality and normal tissue expression concerns reduced practical priority. For the evidence-toxicity visualisation in Fig. 2D, the integrated evidence score was calculated as the weighted sum of network consensus, degree-aware support, seed-dropout stability, pharmacogenomic direction, external context, and direct MTX-combination evidence, normalised by their total weight (0.73). The toxicity-risk score was calculated from the weighted pan-essentiality and normal-expression penalties, normalised by their total weight (0.10). Actionability was retained in the overall MCDA score but was not included on either axis of the Pareto visualisation.

Rank uncertainty was assessed by repeating MCDA across 3000 weight perturbation simulations and by leave-one-component-out analysis, which tested whether candidate rankings were stable to changes in component weights or driven by any single evidence category. Weight perturbation further assessed whether the top-ranked candidate remained stable when different weights were assigned to the MCDA components.

Source-specific p -values and FDR values remained the primary inferential outputs. Because the available evidence sources concentrate signal within overlapping cell-cycle and DNA-damage-repair programs, they do not satisfy the independence assumption required for combined-probability tests such as Fisher's method. Cross-modal support was therefore summarised without combining p -

values: candidates were ranked within each evidence source separately, and the number of sources ranking a candidate among the top three was reported as a descriptive concordance measure. Sources with missing coverage were recorded as unavailable and were neither imputed nor treated as negative evidence. This approach was used for hypothesis ordering only and does not constitute a significance test. It was kept descriptive because candidate selection was not preregistered. To test whether the fixed analysis weights introduced subjective bias, the panel was rescored under two objective weighting schemes. Entropy weights were derived from the dispersion of the normalised component matrix, and analytic hierarchy process weights were obtained from the principal eigenvector of a Saaty pairwise comparison matrix constructed from the stated criterion ordering. The three weighting strategies, together with the 3000-draw Monte Carlo perturbation already described, are compared in **Supplementary Table 1** and **Supplementary Fig. 2**. The fixed weights and their assigned directions are listed in **Supplementary Table 2**.

3. Results

3.1 Network Propagation and Bias-Controlled Candidate Panel Derivation

Computational prioritisation denotes in-silico ranking of candidate hypotheses without functional validation, which is the scope of this study. Preclinical experimental validation denotes wet-laboratory combination testing in cell or tumour models, which is proposed here but not performed. Clinical therapeutic efficacy denotes patient-level tumour response and survival endpoints, which this study does not address. Terms such as synergy, sensitisation, validation and efficacy are reserved for the level at which the supporting evidence was actually generated. Network propagation across four interaction databases yielded a ranked list of candidate genes with elevated propagation scores relative to the seed distribution. Bias-control analyses were then used to examine whether nominated candidates reflected disease-context seed proximity rather than graph topology, hub connectivity, or seed-placement effects. These results were used to define a hypothesis panel for downstream stress testing, not to claim discovery-level network significance. From this list, a final panel of eight candidates was defined for structured downstream analysis: PLK1, PARP1, PRKDC, TP53, CLIP1, CHEK1, ATR, and WEE1. Selection was deliberately conservative and based on three criteria: recurrent prioritisation across multiple networks, biological relevance to MTX resistance, and translational actionability. Candidates representing distinct mechanistic hypotheses were retained even when their network scores were moderate, to avoid premature narrowing before orthogonal stress tests were applied. Tier labels from earlier exploratory analyses were not used as selection inputs to avoid circularity in downstream ranking. The mech-

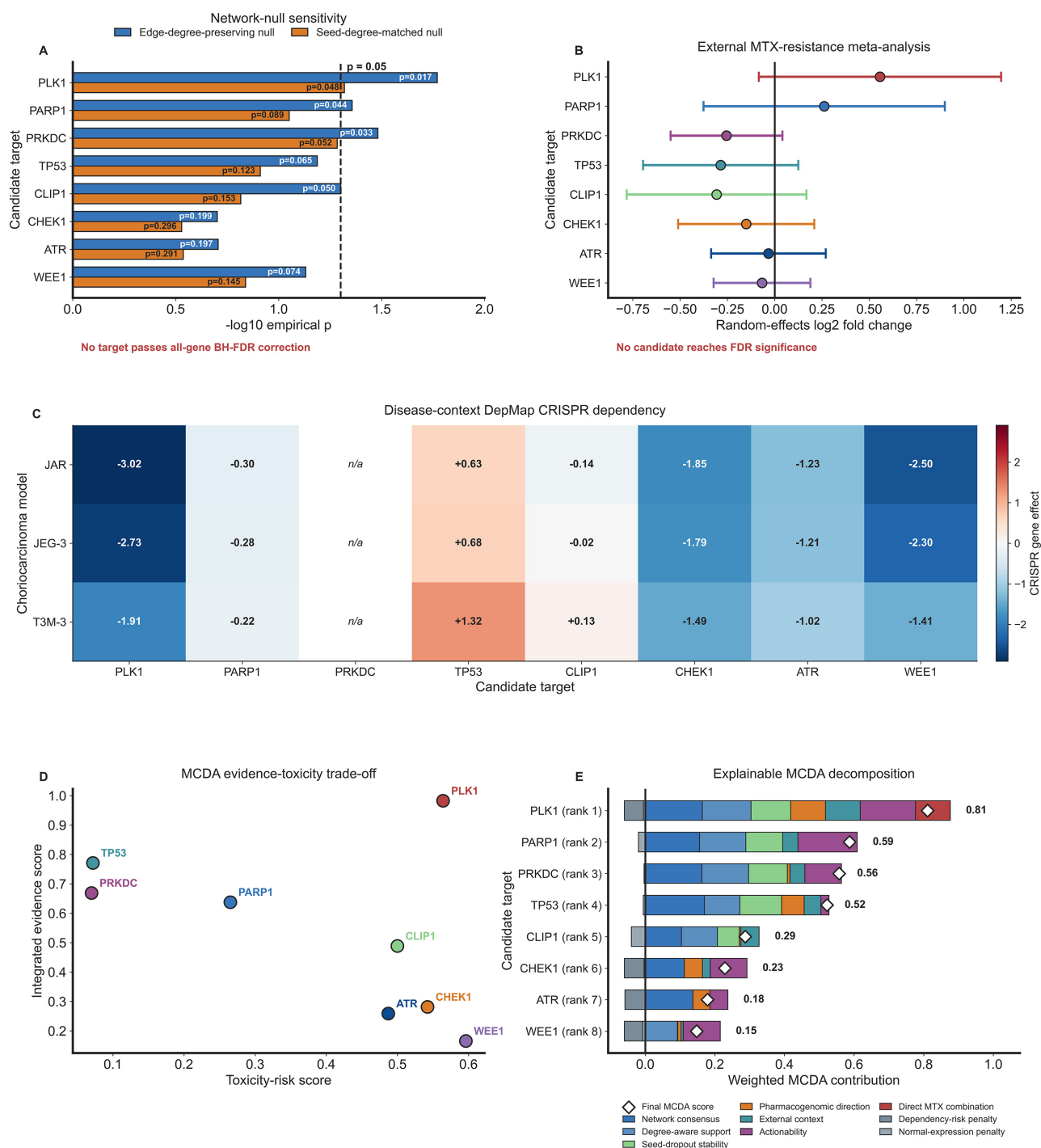


Fig. 2. Integrated evidence prioritisation for MTX-combination assay design. (A) Network-null sensitivity across prioritised targets. (B) External MTX-resistance random-effects meta-analysis. (C) Disease-context DepMap CRISPR dependency heatmap. (D) Transparent multi-criteria decision analysis (MCDA) evidence-toxicity Pareto view, showing the normalised weighted evidence score versus the normalised weighted toxicity-risk score. (E) Explainable weighted-component MCDA decomposition. These panels support experimental triage rather than therapeutic efficacy or disease-selective vulnerability claims.

anistic STRING protein-protein interaction context of the predefined eight-target panel is shown in **Supplementary Fig. 3**.

3.2 Multi-Source Evidence Supports PLK1 as the Strongest Candidate for Integrated Prioritisation

The supporting evidence remained constrained by source-specific uncertainty. In the network bias-control

analysis, PLK1, PRKDC, and PARP1 retained nominal empirical support, with PLK1 showing the strongest signal under both the degree-preserving edge-swap null ($p = 0.017$) and the seed-degree-matched null ($p = 0.048$) (Fig. 2A). However, no candidate passed all-gene Benjamini–Hochberg FDR correction across the 10,746-gene universe, indicating that the network results supported candidate prioritisation rather than discovery-level significance. Detailed global network-null results for all final candidates are provided in **Supplementary Table 3**.

External MTX-resistance meta-analysis likewise provided limited target-level statistical support. PLK1 and PARP1 showed directionally positive pooled estimates, but no candidate reached FDR significance across the five non-trophoblastic MTX-resistance datasets (**Supplementary Table 4**; Fig. 2B). For PLK1, the pooled log₂ fold change was 0.557, with a 95% confidence interval spanning zero (−0.084 to 1.197; BH-FDR, 0.364) and substantial between-study heterogeneity ($I^2 = 88.9\%$). These findings indicate that single-gene expression changes were not sufficiently consistent across heterogeneous MTX-resistance models to support target-level transcriptomic significance. At the pathway level, preranked enrichment analysis recurrently highlighted E2F-target and G2/M-checkpoint programs, although the direction of enrichment varied by disease context. Together, these results provide pathway-level rationale for testing a mitotic-stress strategy while indicating that MTX resistance is not explained by a single pathway or a uniform direction of transcriptional change (**Supplementary Table 5**; **Supplementary Fig. 4**).

PLK1 also showed strong basal dependency across the three DepMap choriocarcinoma models, with a median CRISPR gene-effect score of −2.735 across JAR, JEG-3, and T3M-3 cells (Fig. 2C). Its pan-cancer median gene-effect score was similarly strong (−2.778), however, indicating that PLK1 dependency was broadly observed across cancer models rather than selectively enriched in choriocarcinoma (**Supplementary Table 6**). WEE1, CHEK1, and ATR also showed strong basal dependency, whereas PARP1 showed more modest dependency and PRKDC lacked DepMap CRISPR coverage in the evaluated choriocarcinoma models. None of the three choriocarcinoma models had public PRISM MTX AUC measurements. Accordingly, DepMap CRISPR results were interpreted as evidence of basal target dependency and experimental tractability rather than MTX-specific sensitisation. Model-level and pan-cancer summaries are provided in **Supplementary Table 6**.

Taken together, the network, transcriptomic, and pharmacogenomic analyses did not provide source-specific FDR-significant evidence for any single candidate, but they consistently placed PLK1 among the most strongly supported targets. These complementary signals were therefore integrated with the remaining evidence and translational-constraint layers in the MCDA framework.

3.3 MCDA Uncertainty Analysis Identifies PLK1 as the Lead Experimental Priority

PLK1 ranked first in all 3000 MCDA weight-perturbation simulations (Table 1; Fig. 2), with the highest fixed-weight MCDA score (0.811), a rank 95% interval of 1–1, and a top-three probability of 1.000. Thus, its lead position was completely stable to uncertainty in the assigned component weights. Component-level MCDA profiles for all eight targets are shown in **Supplementary Fig. 5**.

The lead position was also robust to the method used to derive the weights. Rescoring the candidate panel using entropy weights derived from the component matrix and analytic hierarchy process weights derived from a pairwise comparison matrix (consistency ratio, 0.023) placed PLK1 first under all three weighting schemes. Rank agreement with the fixed-weight analysis was complete for the analytic hierarchy process (Spearman ($\rho = 1.000$)) and remained high for entropy weighting (Spearman ($\rho = 0.905$); Kendall ($\tau = 0.786$)). PRKDC remained ranked third under all three schemes, whereas PARP1 and TP53 exchanged ranks 2 and 4 under entropy weighting (**Supplementary Table 1**; **Supplementary Fig. 2**).

PARP1 and PRKDC occupied the next two positions after PLK1, with top-three probabilities of 0.977 and 0.906, respectively, supporting their retention as secondary biomarker-stratified hypotheses. TP53 ranked fourth, with a substantially lower top-three probability of 0.117, whereas CLIP1, CHEK1, ATR, and WEE1 each had top-three probabilities of 0.000. Although CHEK1, ATR, and WEE1 remained biologically relevant checkpoint comparators, their integrated priorities were reduced by pan-essentiality and weaker support across the evidence layers represented in the MCDA. The analysis therefore separated the predefined panel into a leading PLK1 hypothesis, two retained secondary candidates, and a lower-priority comparator group.

The integrated MCDA views further clarify why PLK1 remained the lead despite the source-specific limitations described above. Score decomposition showed that its ranking reflected cumulative support across multiple components rather than dominance by a single evidence source (Fig. 2E). In the evidence-toxicity trade-off view, PLK1 occupied the strongest evidence region while also carrying a visible toxicity-risk signal (Fig. 2D). This caution did not remove PLK1 from the leading position; instead, it identified toxicity-aware and schedule-sensitive experimental design as an important constraint for subsequent validation.

3.4 Exploratory Perturbation Context

GSE192446 was included as an exploratory perturbation reference because it provides vorinostat versus DMSO transcriptional profiles in JEG-3 and JAR cells. This dataset allowed us to examine whether the predefined target panel showed similar reversal context patterns across two trophoblastic cell lines.

Table 1. Transparent MCDA ranking of final MTX-combination target hypotheses.

Gene	MCDA score	Rank	Rank 95% interval	Probability top 3
<i>PLK1</i>	0.811	1	1–1	1.000
<i>PARP1</i>	0.587	2	2–3	0.977
<i>PRKDC</i>	0.557	3	3–4	0.906
<i>TP53</i>	0.522	4	2–4	0.117
<i>CLIP1</i>	0.287	5	5–6	0.000
<i>CHEK1</i>	0.229	6	5–6	0.000
<i>ATR</i>	0.179	7	7–8	0.000
<i>WEE1</i>	0.148	8	7–8	0.000

The table reports the fixed-weight MCDA score, observed rank, rank uncertainty from 3000 weight-perturbation simulations and the probability that each target remains in the top three under weight perturbation.

Across 28,910 genes, vorinostat-associated reversal context scores were positively correlated between the two cell lines (Spearman rho, 0.299; Fig. 3). Because positive values indicate downregulation after vorinostat, the upper right quadrant represents concordant downregulation in both cell lines. Among the predefined eight targets, CLIP1 and TP53 were positioned in this concordant downregulation quadrant. PLK1, PRKDC, CHEK1, ATR and WEE1 showed partial or cell-line-specific patterns, while PARP1 did not show a positive reversal context score in either cell line.

This analysis is descriptive by design. GSE192446 measures transcriptional response to vorinostat, not MTX combination viability or resensitisation of an MTX-resistant phenotype. Therefore, Fig. 3 should be interpreted as a perturbation context visualisation, not as evidence of validated MTX collateral sensitivity.

3.5 Normal Placenta Projection

Within the shared HE8W source prefix, median MTX-resistance module scores were lowest in EVT cells (−0.228, $n = 440$), intermediate in CTB (−0.042, $n = 248$) and STR cells (0.086, $n = 615$), and highest in STB cells (0.342, $n = 64$). Although score distributions overlapped across cell types, the MTX resistance signature was not preferentially associated with an EVT-like transcriptional state (Fig. 4). Cell-level associations were reported descriptively because independent source-prefix replication was insufficient. This pattern is consistent with the proliferative architecture of the trophoblast lineage rather than being an unexplained negative result. Extravillous trophoblast occupies an invasive but comparatively low-proliferative state in this reference (median proliferation score −0.977, versus −0.116 in cytotrophoblast and 0.130 in stromal cells), whereas the MTX-resistance module derived from JEG-3R is weighted toward cell-cycle and DNA-damage-repair programs. Reduced module scores in extravillous trophoblast are therefore the expected consequence of lineage proliferative state. Full HE8W-only cell-type contrasts are provided in **Supplementary Table 7**.

This single-cell projection produced a negative result, which limits a tempting but unsupported biological story. If the resistance signature had mapped strongly to EVT cells, it might have suggested a lineage-state interpretation. Instead, the shared-source analysis showed lower resistance scores in EVT than in cytotrophoblast and syncytiotrophoblast cells. The normal-placenta reference therefore provides a contextual stress test rather than a positive disease model. Corroboration in an MTX-challenged trophoblast single-cell dataset would be the appropriate next test, and we searched for one. Queries of the Gene Expression Omnibus and of ArrayExpress combining MTX with trophoblast, choriocarcinoma or placenta and with single-cell terms returned no MTX-challenged primary trophoblast or choriocarcinoma single-cell dataset. The nearest available resource, GSE300458, profiles MTX-resistant JAR choriocarcinoma cells but is bulk RNA-seq rather than single-cell and was released after the present analysis was completed; it is noted here as available context and is not incorporated into any analysis. Publicly available trophoblast single-cell atlases remain drug-naïve, so no existing dataset can currently corroborate a resistance signature at single-cell resolution. This absence is itself the gap that motivates computational prioritisation, and generating such data is included in the proposed roadmap.

The predefined eight gene sensitivity analysis in E-MTAB-6701 confirmed the same directional result (**Supplementary Fig. 6**). Cell-type descriptive summaries of the predefined eight-gene score, proliferation score, and proliferation-adjusted residual are provided in **Supplementary Table 8**. After pseudobulking 14,034 annotated trophoblast cells across seven individuals, the median paired score difference for EVT versus cytotrophoblast was −0.181 ($n = 7$; Wilcoxon $p = 0.0156$; BH-FDR across paired contrasts = 0.0893). The proliferation-adjusted small-sample sensitivity model also retained a negative EVT coefficient (−0.196). The predefined panel score and its residual after adjustment for proliferation and donor indicators did not form an EVT-high region on the atlas embedding. Cell-level overlays were used for visualisation

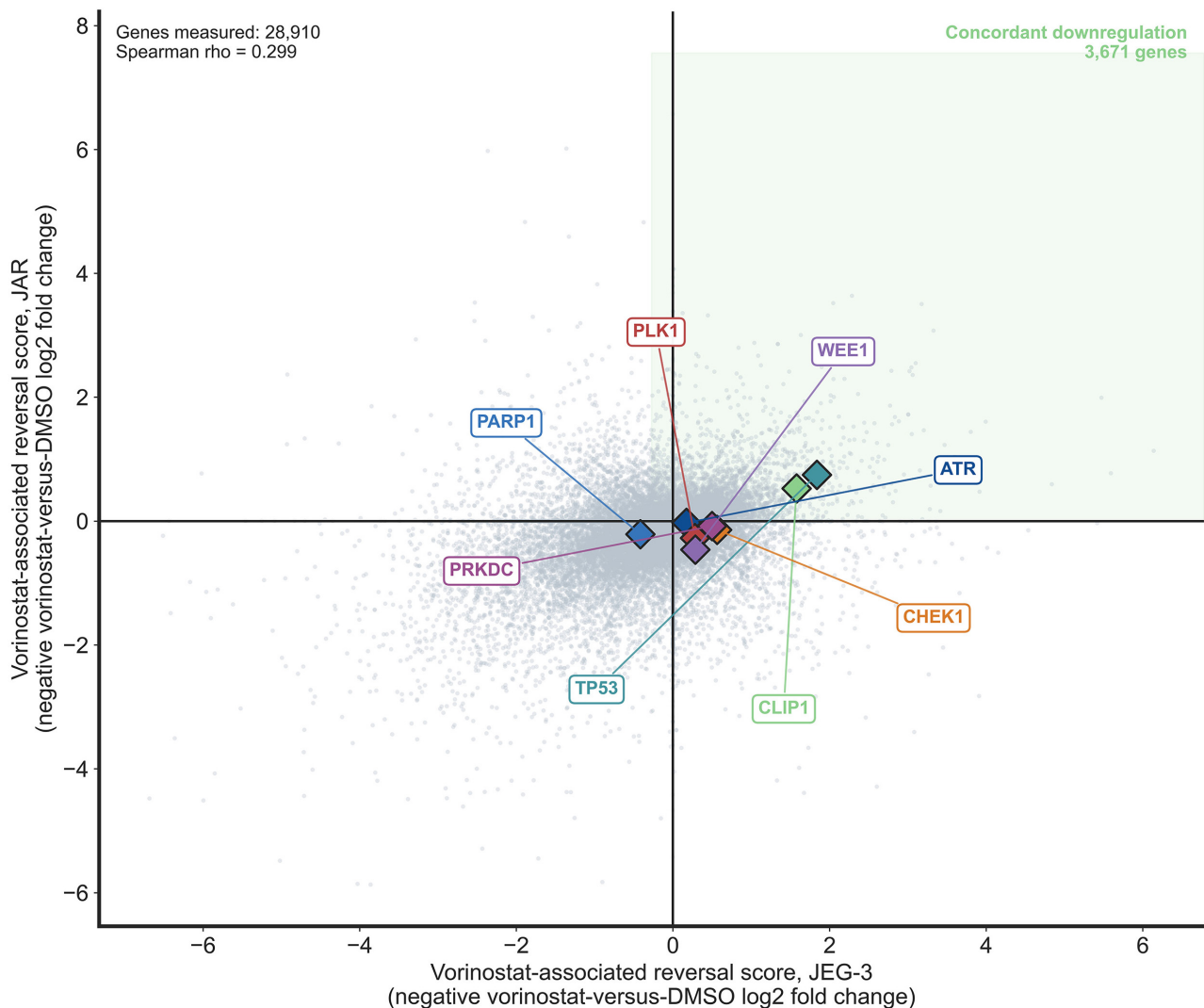


Fig. 3. GSE192446 perturbation-context phenotypic reversal scatter. Each pale point is a measured gene. Axes show the negative vorinostat-versus-DMSO log₂ fold change in JEG-3 and JAR cells, so positive values indicate vorinostat-associated downregulation. Diamonds identify the predefined eight-target panel. The upper-right quadrant marks concordant downregulation context. This visualisation does not establish MTX collateral sensitivity or therapeutic reversal.

only, whereas donor level pseudobulk contrasts remained the primary analysis (**Supplementary Fig. 7**). Donor-level estimates for the predefined panel are given in **Supplementary Table 9**.

3.6 Drug Combination Evidence

After DrugComb records were collapsed to independent units and MTX was separated from other antifolates, only 1 independent MTX plus PLK1-inhibitor unit had positive ZIP synergy (median ZIP, 11.916). This single observation derives from the U-HO1 cell line, which is not of trophoblastic origin, further limiting its disease relevance. PARP-class combinations had broader coverage, with 79 independent units across 40 cell lines and two drug pairs, but the aggregate median ZIP synergy score across available MTX records was not supportive (median ZIP, -0.466).

No direct MTX-combination evidence was available for PRKDC. Detailed collapsed MTX-combination evidence is provided in **Supplementary Table 10**.

This analysis prevents a misleading conclusion that public combination screens already validate the proposed strategy. The PLK1-class observation is directionally encouraging because it involves MTX itself and has a positive ZIP score, but it is a single independent unit. The PARP-class result is almost the inverse: coverage is broader, but the aggregate MTX-combination signal is not favourable. For PRKDC, the absence of direct public MTX-combination evidence means that any DNA-PK hypothesis must begin as a mechanistic experiment rather than as screen-supported repurposing.

An exploratory TDC DrugSyn-based prioritisation layer was added to order candidate-level dose matrix assays

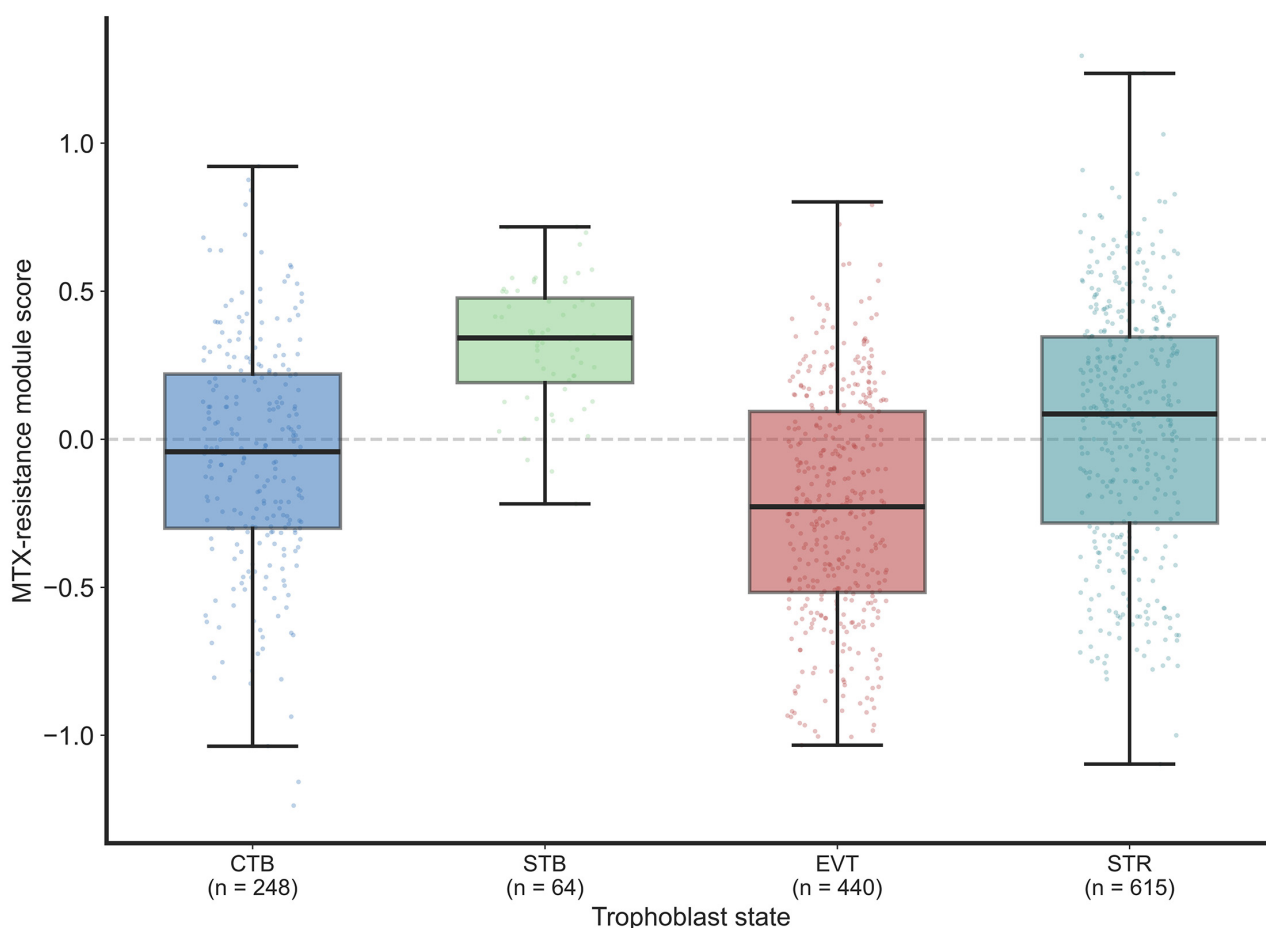


Fig. 4. Normal-placenta single-cell context projection within the shared HE8W source prefix. The MTX-resistance module score was calculated from the top 100 upregulated minus the top 100 downregulated GSE88873 JEG-3R genes using expression-bin-matched control correction. The score is lower in extravillous trophoblast than in cytotrophoblast and syncytiotrophoblast cells. Distributions are descriptive because independent source-prefix replication is insufficient.

(**Supplementary Table 11**; Fig. 5B). Model performance decreased under the more stringent cold drug pair split compared with the random row split. In the RNA hybrid model, ZIP Spearman rho decreased from 0.513 to 0.384, while Bliss Spearman rho decreased from 0.447 to 0.256. None of the six MTX candidate projections produced a strong predicted ZIP signal relative to model spread. Peposertib had the highest projected ZIP score (0.721; tree-level standard deviation, 3.990), followed by talazoparib (0.322; tree-level standard deviation, 3.439). These estimates were limited by domain mismatch. None of the six candidate compounds appears in the TDC training set (exact-structure match absent for all six; nearest-neighbour Tanimoto similarity 0.40 to 0.51), and JEG-3 lies outside the NCI-60 training domain. Critically, the projected values are smaller than the model's own dispersion: the highest projected ZIP score is accompanied by a tree-level standard deviation of 3.990, so the predicted signal lies within model noise and does not support compound-level discrimination. This layer therefore documents an attempted analysis whose predictive res-

olution proved insufficient, and it does not contribute to candidate prioritisation.

3.7 RNA Expression Window

PLK1 median expression was 51.09 TPM across the three DepMap choriocarcinoma models, compared with 30.2 nTPM in Human Protein Atlas bone marrow and 40.9 nTPM in thymus, the highest selected critical normal tissue. PLK1 expression in the tumour models was 1.69-fold higher than the bone marrow value and 1.25-fold higher than the highest selected critical normal tissue value. This narrow RNA expression window reflected relatively high PLK1 expression in proliferative normal tissues, which can be explained by its role as a core mitotic regulator. This finding highlighted the need for toxicity-aware validation in normal proliferating cell contexts.

The expression window result changes how PLK1 should be tested. A narrow tumour-to-normal RNA margin does not invalidate PLK1 as a preclinical target, but it makes continuous high-intensity exposure less attrac-

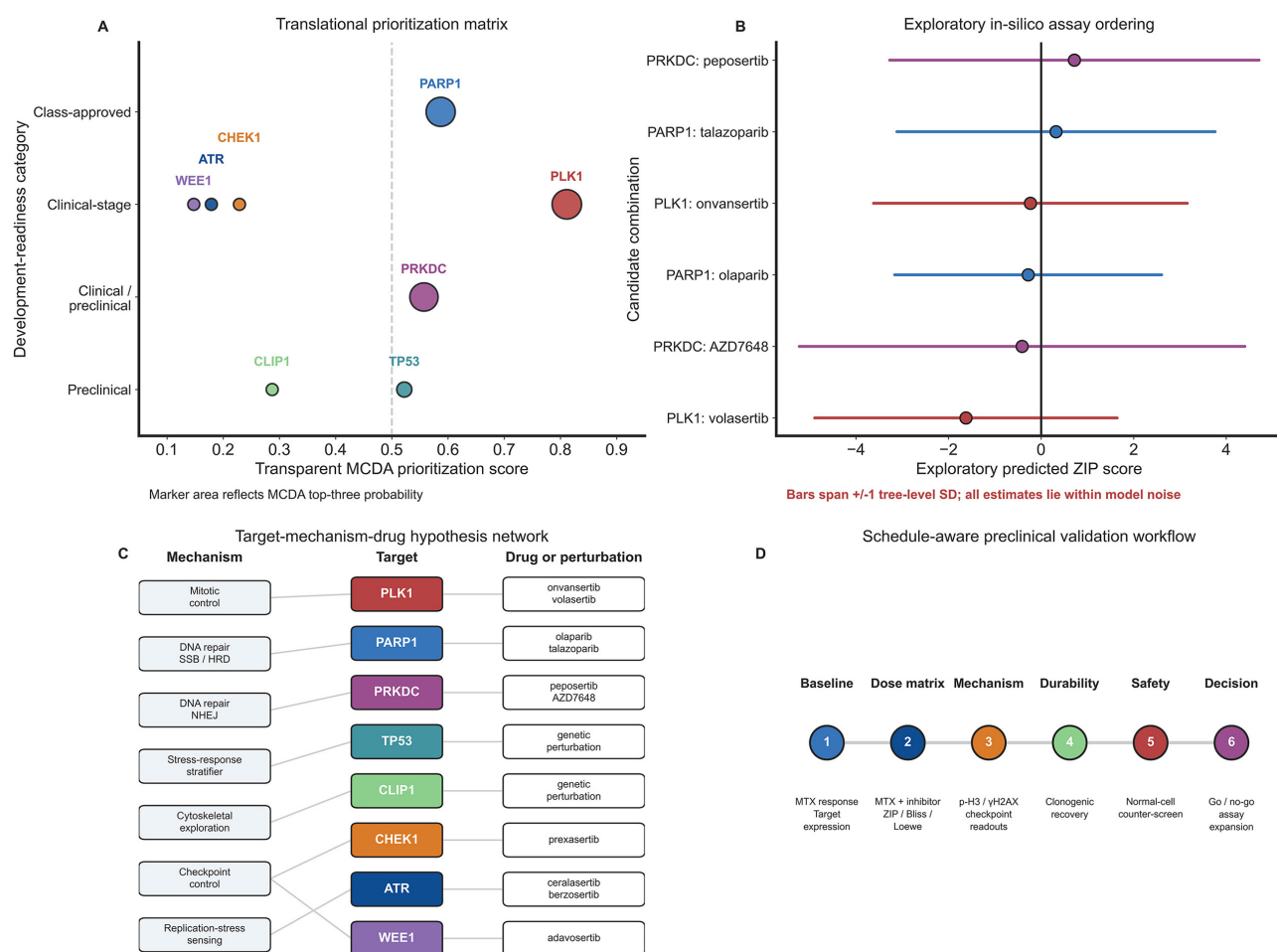


Fig. 5. Translational roadmap for staged MTX-combination testing. (A) Translational prioritisation bubble matrix. Development readiness reflects the highest documented stage of representative target-directed agents: class-approved, clinical-stage, mixed clinical/preclinical, or preclinical/genetic-perturbation only, based on published clinical-development literature and public trial/regulatory information. (B) Exploratory in-silico MTX-combination assay ordering. (C) Target-mechanism-drug hypothesis network. (D) Schedule-aware preclinical validation workflow. Drug links and projected synergy estimates define testable hypotheses, not validated combinations or predicted clinical benefit.

tive. A more defensible design would test MTX pretreatment followed by intermittent PLK1 inhibition, measure mitotic arrest and apoptosis, and include marrow-relevant or trophoblast-like normal-cell controls. This design directly addresses the main translational liability identified by the public data.

The expression window differed across secondary candidates. PARP1 showed less favourable normal tissue RNA context because its highest selected critical normal tissue expression exceeded its tumour model expression. PRKDC showed a wider RNA expression window, although its broader public evidence remained limited.

The cross-platform RNA expression window proxy is shown in **Supplementary Fig. 8**. Detailed values are provided in **Supplementary Table 12**.

3.8 Cross-Modal Rank Concordance Identifies PLK1 as the Most Consistently Supported Candidate

No individual public data modality provided source-specific FDR support for any candidate. Rather than combining source-specific p -values into an omnibus statistic, which would require an assumption of statistical independence that these sources do not satisfy, candidates were compared by their relative rank within each modality, and agreement across modalities was reported descriptively. PLK1 was ranked first by all four evidence sources: the edge-degree-preserving network null ($p = 0.017$), the seed-degree-matched network null ($p = 0.048$), the adjusted DepMap analysis ($p = 0.081$; **Supplementary Table 13**) and the external transcriptomic random-effects meta-analysis ($p = 0.088$). No other candidate achieved this agreement (**Supplementary Table 14**).

PARP1 and TP53 were each ranked among the top three by two of four sources, and the remaining candidates showed no consistent cross-modal agreement. This concordance is descriptive. It indicates that the same candidate is repeatedly favoured by methodologically distinct analyses, which supports experimental ranking; it does not constitute a combined significance test, and the underlying source-specific results remain non-significant after FDR correction.

PRKDC ranked second in all three evidence sources for which it had coverage, but it lacks DepMap CRISPR gene-effect data in the evaluated models. It is therefore reported with an explicit missing-coverage flag and is not treated as rank-comparable with candidates assessed across all four sources. Missing public-data coverage, rather than weak evidence, limits its comparability.

3.9 Focused Validation Roadmap

The integrated evidence was translated into a staged preclinical validation roadmap (Fig. 5). PLK1 occupied the highest-priority testing region because it combined the highest MCDA priority with clinically developed inhibitor candidates, including onvansertib and volasertib (Fig. 5A). PARP1 and PRKDC were retained as secondary experimental arms, represented by olaparib and talazoparib for PARP1 and pemosertib and AZD7648 for PRKDC. WEE1, ATR, and CHEK1 remained lower-priority checkpoint comparators, whereas CLIP1 and TP53 were represented as genetic perturbation hypotheses rather than direct small-molecule strategies.

The exploratory in-silico assay-ordering analysis was retained as a secondary guide and did not override the evidence-based ranking (Fig. 5B). The target–mechanism–drug network organised the candidate panel into mitotic-control, DNA-repair, checkpoint/replication-stress, and genetic-perturbation arms (Fig. 5C), which were mapped onto a common staged workflow comprising baseline MTX-response assessment, combination testing, mechanistic readouts, durability assessment, and normal-cell counterscreening before assay expansion (Fig. 5D). The complete preclinical validation roadmap is provided in **Supplementary Table 15**.

4. Discussion

4.1 Candidate Panel Interpretation

The eight-candidate panel was defined conservatively as a rank experimental roadmap (Table 1). PLK1 was designated the lead hypothesis on the basis of complete rank stability across all weight-perturbation simulations and consistent multi-source evidence convergence.

PARP1 and PRKDC remain useful secondary hypotheses but should not be presented as equivalent to PLK1. PARP1 has clinically accessible inhibitors, yet the aggregate DrugComb signal is non-supportive. PRKDC is mechanistically plausible, but public pharmacogenomic and di-

rect combination evidence are insufficient. Both require biomarker-stratified testing.

Clinical PARP-inhibitor experience and preclinical/early clinical DNA-PK inhibitor studies support the feasibility of these drug classes, but they do not validate MTX-combination activity in choriocarcinoma.

The PARP1 result shows why actionability is not enough. PARP inhibitors are clinically mature and experimentally accessible, but the public MTX-combination evidence did not show a favourable aggregate signal. This does not rule out a stratified PARP1 hypothesis, especially in HRD-like or RAD51-low contexts, but it argues against making PARP1 the default first experiment. PRKDC presents the opposite problem: the biology is coherent for DNA double-strand break repair and non-homologous end joining, but public disease-context coverage is thin. PRKDC is therefore better handled as a smaller mechanistic arm than as public-data-supported repurposing.

4.2 Biological and Clinical Rationale for PLK1 Inhibition

This bias-controlled public-data analysis identifies PLK1 inhibition as the leading MTX-combination hypothesis for experimental testing in choriocarcinoma. The stability of this prioritisation across varied evidence weightings suggests that PLK1's lead position was not driven by a single scoring assumption. The biological rationale for this result lies in the intersection between antifolate-induced nucleotide stress, DNA-damage response, and mitotic recovery.

MTX is an antifolate antimetabolite that interferes with folate-dependent nucleotide synthesis, thereby preventing DNA synthesis in proliferating cells. In choriocarcinoma models, MTX exposure has been shown to induce DNA strand breaks and suppress RAD51-associated homologous recombination repair, linking antifolate pressure to DNA damage response biology [32]. Thus, these effects show that MTX can limit choriocarcinoma cell proliferation by creating nucleotide stress and DNA damage pressure.

However, the effect of MTX is not uniform across all tumour cells. A fraction of cells may be eliminated, whereas others can persist by entering a more tolerant state in which proliferation is delayed and repair pathways are reinforced. In MTX-resistant choriocarcinoma models, Georgiou et al. [10] showed that resistant cells were associated with cell cycle and DNA damage repair programs, including G1 cell cycle delay and ATR-dependent nonhomologous end joining. MTX-resistant choriocarcinoma cells may also avoid apoptosis by shifting toward protective autophagy, a stress-tolerance program that helps residual cells survive MTX pressure. Consistently, Shen et al. [33] reported that MTX-resistant JEG-3 cells showed reduced apoptosis with increased protective autophagy, describing this as a switch from ER stress-induced apoptosis to autophagy through ROS-mediated JNK/p62 signalling.

PLK1 provides a mechanistic bridge from MTX-induced damage tolerance to mitotic progression. Prior work has shown that PLK1 promotes recovery from DNA damage-induced arrest and enables cells to re-enter mitosis after damage checkpoint activation [34,35]. PLK1 activity is itself suppressed during the damage response through loss of Aurora A recruitment by Bora. Pharmacologic PLK1 inhibition therefore provides a way to interrupt the post-stress mitotic recovery step that MTX-tolerant cells may require to continue proliferating. This is consistent with broader PLK1 inhibitor literature. Gutteridge et al. [36] reviewed PLK1 inhibitors as cancer therapeutics and noted that PLK1 inhibition can cause mitotic block and apoptosis in cells with high mitotic activity, while also emphasising that combination strategies may be needed because of resistance to monotherapy and limited clinical translation. At the therapeutic strategy level, PLK1-based combination therapies have also been reported to produce stronger anti-cancer effects and show potential to overcome resistance compared with monotherapy [37]. In ovarian cancer models, Huo et al. [38] identified the PLK1 inhibitor BI 2536 as an effective anti-ovarian cancer agent that inhibited proliferation, induced cell cycle arrest, and promoted apoptosis and pyroptosis. In cervical cancer models, volasertib further supported a PLK1-directed combination strategy by reducing tumour growth and enhancing the antitumour effect of cisplatin-based treatment in both *in vitro* and *in vivo* experiments [39]. More recently, two studies published in 2025 have extended this rationale to onvansertib, a selective PLK1 inhibitor. Sinha et al. [40] showed that onvansertib reduced malignant cell growth, impaired invasive behaviour, and suppressed tumour growth in a genetically engineered mouse model of endometrioid endometrial cancer. In parallel, Haag et al. [41] reported that onvansertib inhibited proliferation and increased paclitaxel sensitivity in uterine serous cancer cells. Together, these data are particularly relevant because they place PLK1 inhibition within uterine malignancy models, a gynaecologic disease context that is biologically and anatomically close to trophoblastic tumours such as choriocarcinoma. These findings support the emerging view that PLK1 inhibition may be most promising when developed as part of rational combination strategies in female reproductive tract malignancies, thereby supporting the plausibility of testing as a post-MTX mitotic blockade in choriocarcinoma models.

4.3 Methodological Contribution

Current therapeutic strategies for MTX-resistant GTN can be broadly grouped into conventional salvage chemotherapy, immunotherapy, and molecularly targeted therapy, with surgery or other lesion-directed interventions reserved for selected cases. Several individual studies and reviews have suggested that treatment strategies for chemotherapy-resistant GTN are expanding beyond conventional salvage chemotherapy, particularly through im-

mune checkpoint inhibition and molecularly targeted therapy [42,43,44]. Immunotherapy has shown encouraging clinical activity in selected resistant or relapsed GTN cases, with pembrolizumab, avelumab, and PD-1-based combination regimens showing meaningful results [45,46,47]. Molecularly targeted therapy has also attracted growing interest, with VEGFR-directed strategies, anti-endoglin targeting, and other targetable pathways showing preliminary promise in refractory GTN and choriocarcinoma [10,48,49,50]. However, the available evidence remains fragmented across disease subtypes, treatment lines, cohort sizes, and outcome measures. This fragmentation makes it difficult to determine which therapeutic hypothesis should be prioritised for MTX-resistant choriocarcinoma, especially when direct disease-specific combination evidence is sparse. Therefore, a systematic screening approach is needed to compare candidate strategies across heterogeneous evidence layers while explicitly controlling for these sources of bias.

The present study addresses this methodological gap by converting dispersed public evidence into a multi-layered screening framework. Its methodological value lies in forcing each candidate hypothesis to pass through complementary evidence filters, including disease-context molecular signals, network-level bias controls, pharmacogenomic stress tests, external expression context, trophoblast biological relevance, and normal-tissue constraints. This design is vital because a ranking based only on network proximity, druggability or dependency would probably promote familiar cell-cycle and DNA-repair targets, but it would say little about whether the signal reflects network structure, pan-essentiality or sparse combination evidence. Separating prioritisation from validation makes the evidence easier to audit and helps define the boundary between experimentally testable hypotheses and unsupported therapeutic claims. That distinction is useful for rare cancers and for resistance questions where matched datasets are unlikely to appear quickly. Waiting for large public cohorts may not be realistic, but moving from a single omics signal directly to a therapeutic claim is not justified. A transparent evidence-ranking framework helps bridge discovery and validation, and prevents exploratory evidence from being overstated. Set against recent multi-criteria prioritisation work, the contribution here is the combination rather than any single element (**Supplementary Table 16**). Resources such as the Open Targets Platform aggregate evidence at far greater scale and include safety annotation, but do not quantify uncertainty in the resulting prioritisation [51]. Network-based frameworks that weigh therapeutic efficacy against adverse-reaction potential [52], and co-essentiality approaches that apply degree-controlled permutation testing [53], each implement one of the controls used here but not the others. Pipelines built on CRISPR dependency data commonly exclude common-essential genes yet rely on sequential filtering rather than

an explicit weighted model [54], and recent frameworks that penalise expression in critical normal organs do not report ranking confidence [55]. Three features distinguish the present workflow: six heterogeneous evidence types are combined in one quantitative model; bias control operates at every layer, including two degree-preserving permutation nulls, all-gene FDR correction across 10,746 genes, weight perturbation, and a rank-based cross-modal concordance analysis that avoids the independence assumption of combined-probability tests; and the decision model is explicitly bidirectional, penalising pan-essentiality and normal-tissue expression rather than rewarding tumour-directed signal alone. That penalty is consequential here, since it is what exposes the narrow PLK1 expression window and drives the intermittent rather than continuous dosing schedule we propose. Consistent with this broader principle, recent oncology work has shown that integrating bulk and single-cell transcriptomic evidence with external validation can support biomarker development for treatment-response prediction [56].

4.4 Limitations

The originating SILAC comparison lacks independent biological replicates and is therefore unsuitable for inferential omics discovery. Disease-specific public data remain sparse: the GSE88873 sensitive arm contains one sample, none of the three choriocarcinoma DepMap models have public PRISM MTX-AUC measurements and the positive DrugComb PLK1 observation is a single independent unit. External MTX-resistance datasets are largely non-trophoblastic and heterogeneous. No candidate passes the global network FDR correction or source-specific pharmacogenomic FDR correction. Cross-modal agreement is descriptive and based on relative ranks; it is not a combined significance test, and the underlying source-specific results remain non-significant after FDR correction. The normal-placenta single-cell reference does not support patient-level inference. The TDC DrugSyn-based estimates also remain exploratory because JEG-3 lies outside the NCI-60 training domain, its projected RNA norm slightly exceeds the training-domain maximum and none of the proposed inhibitors is an exact training compound. Finally, cross-platform RNA ratios do not model drug exposure, dose-response or clinical toxicity. Direct MTX-combination assays in paired sensitive and resistant trophoblastic models remain necessary.

Several limitations follow from the deliberate use of public data. The analysis cannot account for patient-level treatment history, tumour purity, pharmacokinetics, drug scheduling or acquired resistance mechanisms that are absent from the source datasets. The network analyses depend on curated interaction resources that are biased toward well-studied genes. The external transcriptomic analysis pools non-trophoblastic MTX-resistance models and is therefore best interpreted as a pathway context. The

normal-tissue RNA comparison crosses platforms and does not include protein abundance, target occupancy or tissue-specific adverse-event data. Each of these constraints has a specific consequence for interpretation rather than being a generic caveat. First, no patient-derived choriocarcinoma line and no *in vivo* xenograft model were available, so no statement can be made about *in vivo* activity, exposure or tolerability; the roadmap therefore places *in vivo* testing before any translational claim. Second, the public datasets differ in tissue origin, sequencing platform and MTX exposure schedule, and this heterogeneity is quantitatively visible as between-study inconsistency reaching an I^2 of 95.3% in the random-effects meta-analysis; it is the principal reason no candidate reaches FDR significance, and it is why the transcriptomic layer is interpreted as pathway-level context rather than target-level evidence. Third, normal-tissue nTPM values and proteomic abundance readouts are not quantitatively commensurable, so the expression window functions only as an ordinal warning layer and precludes therapeutic-index modelling. Fourth, all conclusions are restricted to MTX-resistant choriocarcinoma; the seed evidence derives from a single MTX-resistant choriocarcinoma model, and no extension to high-risk GTN, placental-site or epithelioid trophoblastic tumour, or other trophoblastic subtypes is supported. In addition, this study does not include *in vivo* models. The proposed PLK1 strategy and the secondary DNA-damage-response arms, including PARP1/PRKDC, still require mouse or other appropriate *in vivo* testing to assess systemic toxicity, exposure, schedule feasibility and whether an apparent MTX-combination effect can be achieved at tolerable doses, with WEE1 retained only as a lower-priority exploratory comparator. These limitations do not negate the prioritisation result, but they define the boundary between computational triage and experimental validation. A further question is how far the framework itself transfers beyond this disease. No step in the workflow is specific to trophoblastic tumours: network propagation, both permutation nulls, the MCDA layer and the uncertainty analyses operate on any seed set, and the pharmacogenomic, transcriptomic, drug-combination and normal-tissue layers draw on species-level resources. The binding requirement is the seed itself, namely a matched drug-sensitive versus drug-resistant proteomic and phosphoproteomic comparison in the disease of interest. **Supplementary Table 17** sets out this requirement layer by layer alongside the current public availability of each resource for other gynaecologic malignancies. For MTX specifically, that seed does not exist outside trophoblastic disease, because MTX is not a standard agent in ovarian, endometrial or cervical cancer and matched resistant models have therefore not been generated. Prospective application to a second disease context, using an agent for which such paired resources exist, is the natural next step for this framework.

4.5 Clinical Implications

The principal translational constraint on the PLK1 hypothesis is its lack of disease-selective dependency together with the narrow RNA expression window observed in proliferative normal tissues. PLK1 should therefore be regarded not simply as a target-selection hypothesis, but as a schedule-dependent combination hypothesis. MTX may first impose nucleotide depletion and replication stress, after which PLK1 inhibition could interfere with recovery and subsequent mitotic progression. Sequential MTX exposure followed by intermittent PLK1 inhibition is therefore a more defensible starting design than continuous concurrent treatment, although the optimal sequence, interval, and dose intensity cannot be determined from the present public-data analysis and require direct experimental measurement.

A focused validation study should consequently extend beyond short-term viability. For PLK1, evidence of combination benefit should be accompanied by pharmacodynamic confirmation of PLK1 pathway inhibition, mitotic-arrest or apoptosis readouts, and assessment of durable response using clonogenic or recovery assays. Experiments should include paired MTX-sensitive and MTX-resistant or resistance-enriched trophoblastic models, together with a normal proliferating-cell comparator. Pharmacodynamic measurements should be collected under the same schedules used for combination testing so that mechanism and response can be interpreted together.

The secondary hypotheses require different experimental strategies. PARP1 should be evaluated preferentially in HRD-like or RAD51-low contexts because the available public MTX-combination evidence is mixed and does not support assuming a general synergistic effect. For PRKDC, the principal mechanistic question is whether MTX-induced replication stress generates a DNA-PK-dependent repair state in trophoblastic models; appropriate validation would therefore include DNA-damage readouts such as γ H2AX, 53BP1 foci, and clonogenic recovery. These biomarker- and mechanism-informed designs reflect the different uncertainties underlying the secondary candidates rather than treating them as equivalent alternatives to PLK1.

Toxicity represents an equally important go/no-go criterion. Clinically evaluated PLK1 inhibitors, including volasertib, have shown substantial haematological toxicity, particularly neutropenia and thrombocytopenia [57]. The normal-tissue counterscreen independently highlights the same concern: PLK1 expression in the choriocarcinoma models was only 1.69-fold higher than that in bone marrow and 1.25-fold higher than the highest selected critical normal tissue, representing the narrowest expression window among the prioritised candidates. Thus, tolerability rather than target engagement may ultimately define the feasible exposure and schedule. Preclinical evaluation should therefore include marrow-relevant or other rapidly proliferating

normal-cell controls and explicitly report the exposure margin achieved.

Reproductive toxicity is also relevant because GTN predominantly affects women of reproductive age, for whom preservation of fertility is frequently an important treatment objective. As a core regulator of mitosis, PLK1 inhibition would require careful evaluation of gonadal toxicity and reproductive safety before any translational development. Together, these considerations support intermittent rather than sustained PLK1 exposure and reinforce the need for toxicity-aware scheduling rather than simply maximising target inhibition.

The single-cell analyses further constrain the biological interpretation of the proposed strategy. The MTX-resistance signature did not preferentially map to EVT cells in the normal-placenta reference, and neither the GSE89497 resistance score nor the predefined eight-gene score in the donor-level E-MTAB-6701 analysis supported an EVT-high state. The present data therefore do not support describing MTX resistance as EVT-like lineage reprogramming or proposing that PLK1 maintains such a state. Instead, the single-cell results narrow the interpretation to drug-response prioritisation and illustrate the value of contextual analyses in excluding otherwise plausible but unsupported mechanisms.

Overall, the validation roadmap converts the computational ranking into a set of falsifiable experimental questions. PLK1 should be tested first using schedule-aware, mechanism-informed, and toxicity-controlled combination experiments, whereas PARP1 and PRKDC should remain smaller biomarker- or mechanism-stratified arms. Advancement beyond this stage should require reproducible combination benefit, mechanistic confirmation, durable response, and an acceptable separation from normal proliferating-cell toxicity.

5. Conclusions

This bias-controlled computational prioritisation framework integrates heterogeneous molecular data to systematically rank therapeutic hypotheses for MTX-resistant choriocarcinoma while explicitly distinguishing computational prioritisation from therapeutic validation. Within this framework, MTX plus PLK1 inhibition emerged as the highest-priority preclinical combination for experimental evaluation, whereas PARP1 and PRKDC remain secondary biomarker-stratified hypotheses. The appropriate next step is a focused experimental study incorporating paired MTX-sensitive and MTX-resistant trophoblastic models, schedule optimisation, pharmacodynamic characterisation, and normal-cell counterscreens.

More broadly, this study demonstrates how heterogeneous molecular evidence can be integrated into a transparent, reproducible, and biologically interpretable computational prioritisation strategy for therapeutic hypothesis generation in data-limited diseases. The central message

is therefore practical: computational analyses do not establish a new therapy, but they can justify a disciplined first experiment. Accordingly, PLK1 should be evaluated as a schedule-dependent, toxicity-aware MTX-combination hypothesis, whereas PARP1 and PRKDC should be retained as secondary mechanistic arms until supported by direct experimental validation.

Availability of Data and Materials

All data analysed in this study were obtained from publicly available resources. GEO accession numbers were GSE88873, GSE192446, GSE16066, GSE16070, GSE16080, GSE16082, GSE16089 and GSE89497. The Vento-Tormo normal first-trimester single-cell atlas was obtained from ArrayExpress accession E-MTAB-6701. Additional public resources included DepMap/PRISM, STRING, OmniPath, BioGRID, Reactome, Human Protein Atlas, DrugComb and the TDC DrugComb DrugSyn resource. The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

Author Contributions

KNL: Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualisation, Writing – original draft, Writing – review & editing. TVV: Formal analysis, Investigation, Visualisation, Validation, Writing – review & editing. NQKL: Conceptualization, Methodology, Funding acquisition, Project administration, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Not applicable.

Acknowledgment

The graphical abstract was created using BioRender (Created in BioRender. Le, K. (2026) <https://BioRender.com/gfpuu0q>). Agreement number: WA2A5WWTa1. The authors would like to thank Can Tho University of Medicine and Pharmacy for its support.

Funding

This research was funded by the National Science and Technology Council, Taiwan [grant number NSTC114-2221-E-038-015].

Conflicts of Interest

Given his role as the Guest Editor, Nguyen Quoc Khanh Le had no involvement in the peer-review of this article and has no access to information regarding its peer

review. Full responsibility for the editorial process for this article was delegated to Yudong Cai.

Declaration of AI and AI-Assisted Technologies in the Writing Process

During the preparation of this work, the authors used ChatGPT-5.5 in order to check spelling and grammar. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at <https://doi.org/10.31083/FBL55527>.

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