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'High-Throughput Analysis of Core Pathways in HeLa Cells: Single-Cell Sequencing and AI-driven Modeling of Multi-Pathway Interaction Networks

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Abstract. HeLa cells, the first successfully cultured human cancer cell line, are pivotal in cancer research, virology, and drug screening. However, their multi-omics heterogeneity and complex cancer-related cascades challenge traditional bulk sequencing, which fails to capture dynamic cell-cell interactions and resolve pathway crosstalk. This review systematically examines single-cell multi-omics technologies (transcriptomics, proteomics, and data integration) and AI-driven network modeling (graph neural networks, deep learning) for decoding HeLa cells' core pathways and metastasis mechanisms. It reveals single-cell-level lipid metabolic heterogeneity in cell cycle regulation, dynamic coupling of Ras/NF-κB and PI3K/AKT pathways, and HPV protein-accelerated cell cycles. Glycolysis and oxidative phosphorylation synergize to meet energy demands during metabolic reprogramming, while lactate promotes invasion. Apoptosis resistance involves high anti-apoptotic gene expression and endoplasmic reticulum stress proteins. AI models address data sparsity, predicting metabolic pathways, drug responses, and cell communication networks. Future research should develop high-sensitivity spatial multi-omics, HeLa-specific AI models, and organoid platforms to advance precision medicine.

Keywords: HeLa cells, single-cell multi-omics, AI-driven network modeling, cell cycle regulation, apoptosis resistance.

1. Introduction

The HeLa cell line, the first successfully cultured human cell line, has been pivotal in cancer research, virology, drug screening, and toxicity studies due to its stability, immortality, and culturing ease. However, recent research reveals HeLa cells exhibit multi-omics heterogeneity and complex cancer-related cascade reactions [1]. Traditional bulk sequencing methods face critical limitations: (1) inability to capture dynamic cell-cell interactions; (2) limited resolution for complex pathway crosstalk; (3) lack of spatiotemporal resolution; and (4) high data noise/sparsity [2–4]. These bottlenecks hinder precise decoding of molecular mechanisms underlying HeLa cells' malignant phenotypes, particularly in tumor microenvironment interactions and metastasis cascades.

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To address these, single-cell multi-omics sequencing and AI-driven analytics have emerged. Single-cell technologies provide high-resolution genomic/transcriptomic data to unveil cellular heterogeneity, while AI models integrate multi-omics via pre-training/transfer learning to enhance cell type annotation and gene network inference [5]. AI also simulates intercellular signaling to predict drug effects [6] and removes batch effects to unify multi-modal data [2], enabling comprehensive analysis of gene expression, protein abundance, and chromatin accessibility.

This review systematically analyzes HeLa cells' core pathways and metastasis mechanisms using single-cell multi-omics and AI-driven network modeling, aiming to advance cervical cancer precision therapy and biomedical research.

2. Key technologies for HeLa cell pathway analysis

2.1. Construction of single-cell multi-omics profiles

2.1.1. Advances in single-cell transcriptome sequencing

Single-cell transcriptome sequencing isolates cells/nuclei for RNA high-throughput sequencing. scRNA-seq, the gold standard, reveals intra-cellular RNA heterogeneity and tissue cell typing [7]. Since 2009, technologies like Tang method, SMART-Seq, and sci-RNA-seq have improved throughput, cost-efficiency, and low-abundance transcript detection, enabling million-cell analysis per run [7,8]. Innovations include microfluidic-based platforms, third-generation sequencing (TGS), and CRISPR-coupled Perturb-seq [9–11].

Early bulk RNA-seq studies showed HeLa cells overexpress >2,000 proliferation/DNA repair genes and carry nonsynonymous mutations, indicating DNA repair activation despite chromosomal instability [12]. Comparative analysis with normal human epidermal keratinocytes (NHEK) via Illumina revealed distinct expression in cell proliferation and cytoskeletal remodeling [13]. Unlike bulk sequencing, scRNA-seq resolves single-cell heterogeneity, subpopulates cell subsets, and defines functional states [14–16].

Recent scRNA-seq studies on HeLa-CCL2 cells uncovered transcriptomic heterogeneity linked to proliferation and interferon response [15]. Cell cycle analysis reconstructed transcriptional dynamics, revealing S-phase dosage imbalances resolved by coordinated gene replication [16]. SCAN-seq2, a microfluidic full-length scRNA-seq, showed spliceosome inhibitor IGG enriches proliferation/ invasion genes in HeLa cells, offering drug response insights [14].

2.1.2. Single-cell proteomics for signaling dynamics

While scRNA-seq reveals transcriptional complexity, proteins—functional executors—are post-transcriptionally regulated, necessitating single-cell proteomics. This technology directly measures protein abundance/modifications but faces challenges: low single-cell protein content, amplification difficulty, and data analysis complexity [17,18].

Recent advances enable high-throughput HeLa cell proteomics: AM-DMF-SCP identifies ~2,300 proteins/cell with on-chip processing to reduce loss [19]; PiSPA/SC-pSILAC quantify >3,000 proteins; ProteoCHIP EVO 96 combined with mass spectrometry detects >5,000 proteins, and Chip-Tip identifies >6,000 proteins including >200 membrane proteins [17–20].

Functional studies show Chip-Tip detects 168 protein kinases in HeLa cells, with CDK1/MAPK1 enriched, possibly linked to proliferation [17]. PiSPA analysis of migrating HeLa cells found elevated focal adhesion/actin cytoskeleton proteins (Cdc42/Rac1/RhoA) [18]. SC-pSILAC revealed

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cell cycle proteins (CDK1/CDC20) have higher turnover in dividing cells, linking protein metabolism to proliferation [20].

2.1.3. Multi-omics data integration

Single-omics technologies lack systemic insights, driving development of multi-omics integration. Studies combining scRNA-seq, proteomics, and genomics depict HeLa cell heterogeneity, tumor traits, and drug responses [21].

A 13-lab study integrated transcriptomic/proteomic/genomic data from HeLa variants (CCL2/S3/Kyoto) and found extensive copy number variations (CNVs). HeLa CCL2 had 1.87-fold more diploid genes than Kyoto, with protein expression linked to CNVs and turnover changes during passaging [1]. Another study integrated gene/protein/metabolite networks via random walk analysis (RWR), identifying cell cycle regulators (CDK1/SETD1B) and drug resistance mechanisms in 3D-cultured HeLa cells, where HIF1A activation and glycolysis drive 5-FU resistance [22,23].

Emerging technologies like Paired-Damage-seq (DNA damage-transcriptome) and UDA-seq (high-throughput multi-modal) enable the study of DNA damage-chromatin regulation and cross-species cell typing [10]. Future tools like GLUE (chromatin accessibility-DNA methylation-transcriptome) and scSTAP (transcriptome-proteome) will deepen pathway modeling [24–26].

2.2. AI-driven pathway activity quantification

2.2.1. Limitations of traditional pathway analysis

Traditional methods (GSEA/PGSEA) fail in single-cell multi-omics due to high sparsity ("dropouts") and noise. ssGSEA detects only 30% of gene sets at 80% noise, while tools like AUCell misinterpret dropouts as biological inactivity [27–29]. Data integration is hampered by gene set size sensitivity, incomplete pathway databases (e.g., KEGG), and static single-omics analysis [30,31].

2.2.2. Deep learning applications

Graph neural networks (GNNs) excel in pathway prediction by modeling molecular interactions. Baranwal's GCN-RF hybrid predicts metabolic pathways with 95.16% accuracy [32]. GPDRP uses GNN and Graph Transformer to integrate drug structures and gene pathway activity, improving IC50 prediction [33]. Hi-GeoMVP combines GNN and geometry-enhanced GNN to boost drug response prediction accuracy (Pearson r=0.941) [34].

Multi-modal models like STASCAN (spatial transcriptomics-histology) and DeepMPF (heterogeneous network-semantic analysis) enhance pathway resolution [35,36]. PathNetDRP integrates pathways and PPI networks to predict immune checkpoint responses via PageRank [37]. These models enable biological interpretation, such as STASCAN's cell distribution mapping in human heart tissue [36].

3. Core pathway analysis in HeLa cells

3.1. Single-cell characteristics of cell cycle regulatory pathways

The regulation pathways of the cell cycle in HeLa cells exhibit complexity and dynamic characteristics at the single-cell level, which are specifically reflected in the cycle heterogeneity

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observed at single-cell resolution and the diverse dynamic change patterns of the activity of cell cycle-dependent pathways in HeLa cells.

Recent single-cell mass spectrometry imaging techniques suggest the presence of cell cycle-related lipid metabolism heterogeneity in HeLa cells. Through the tapping mode scanning probe electrospray ionization (t-SPESI) technique, single-cell mass spectrometry imaging (SC-MSI) has revealed significant differences in the distribution of lipid molecules between the nucleus and Golgi apparatus regions in HeLa cells [38]. This study shows that the signal intensity of lipid molecules such as phosphatidylcholine (PC) 34:1[M+H] + is 40% lower in the nuclear region than in the Golgi apparatus region, but 2.3 times higher in the nuclear lateral region (where the Golgi apparatus is located). This indicates that lipid metabolism has significant cell cycle-dependent spatial distribution characteristics, suggesting the demand for membrane structure reorganization at different cell cycle stages [38].

In the periodic dynamic regulation of key signaling pathways, the Ras/NF-κB and PI3K/AKT pathways, as well as human papillomavirus (HPV) proteins, play core roles:

Ras/NF-κB pathway: Mitofusin 2 (Mfn2) arrests the cell cycle in the G0/G1 phase by inhibiting the Ras/NF-κB signaling pathway. Studies have shown that overexpression of Mfn2 can significantly downregulate the expression of Ras, Myc, CyclinD1, STAT3, and NF-κBp65 proteins, leading to the inactivation of downstream Ras signals and thus inhibiting the proliferation of HeLa cells [39].

PI3K/AKT pathway: Lancatanine sulfate (LS) induces G0/G1 phase arrest by inhibiting the PI3K/AKT/GSK3β pathway, manifested by the downregulation of CyclinD1 and phosphorylated Rb (p-Rb), and the upregulation of p21 and p53 expression [40]. Mechanistically, LS triggers a decrease in the Bcl-2/Bax ratio, collapse of the mitochondrial membrane potential (MMP), and caspase-9/7/3 cascade activation through the mitochondrial-mediated apoptosis pathway, demonstrating the close coupling between cell cycle arrest and apoptosis signals [40].

Regulation by HPV proteins: The HPV E7 protein promotes the G1/S phase transition by degrading pRb to release E2F, and by binding to p21 and p27 to relieve their inhibition of CDK2, synergistically accelerating the cell cycle process [41]; HPV E6 enhances cell viability by activating the PI3K/Akt/mTOR pathway [41].

3.2. Activity distribution and interaction of pathways related to metabolic reprogramming

The metabolic reprogramming of HeLa cells represents a critical mechanism for these cancer cells to adapt to rapid proliferation and meet survival demands. It may also endow HeLa cells with resistance to certain therapies and accelerate tumor progression. Therefore, understanding the cellular mechanisms underlying metabolic reprogramming in HeLa cells-particularly the core glycolytic and oxidative phosphorylation pathways—will facilitate insights into the dynamic distribution of metabolic activity in HeLa cells and the discovery of novel therapeutic targets.

Glycolytic Pathway: HeLa cells exhibit a classic "Warburg effect," relying on glycolysis for energy production even under aerobic conditions to meet the high ATP demands of proliferation [13,42]. Key regulatory enzymes such as phosphofructokinase (PFK) show significantly upregulated activity, while pyruvate kinase M2 (PKM2) is overexpressed, with its phosphorylation status modulating glycolytic flux [13,42]. Accumulation of the metabolic product lactate promotes epithelial-mesenchymal transition (EMT) by activating the TGF-β/Smad signaling pathway, thereby enhancing the migratory and invasive capabilities of tumor cells [13,43]. Additionally, HPV E6/E7 proteins promote lipid synthesis by regulating transcription factors such as MYC, providing building blocks for cell membrane biogenesis to support rapid cell proliferation [44].

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Oxidative Phosphorylation Pathway: Although baseline activity is low, HeLa cells maintain energy metabolism by balancing glycolysis and oxidative phosphorylation. When glycolysis is inhibited, the activity of mitochondrial respiratory chain complexes is upregulated to compensate for energy demands, demonstrating metabolic flexibility [13]. Concurrently, enhanced pentose phosphate pathway activity generates NADPH to mitigate oxidative stress-induced cellular damage [42].

Coordination of Cell Cycle and Metabolism: Glycolytic activation is closely associated with G1/S phase progression in the cell cycle, ensuring the supply of energy and metabolites at different growth stages [45]. For example, LS-induced cell cycle arrest is accompanied by mitochondrial dysfunction, further confirming the dynamic coupling between metabolic pathways and the cell cycle [40].

3.3. Multi-omics evidence of apoptotic resistance mechanism

The mechanisms of apoptosis resistance in HeLa cells are significantly manifested at multiple omics levels, including transcriptomics, proteomics, and metabolomics. This phenomenon profoundly reveals the complex survival strategies formed by HeLa cells during evolution.

Transcriptomic Characteristics:Upregulated expression of anti-apoptotic protein genes represents a core mechanism. Members of the Bcl-2 family (e.g., Bcl-2, Bcl-xL) block the apoptotic cascade by inhibiting the release of cytochrome c from mitochondria [46]. High expression of cellular FLICE-like inhibitory protein (cFLIP) forms complexes with FADD and caspase-8, preventing caspase-3 activation. Additionally, cFLIP synergizes with the NF-κB pathway to further upregulate its expression in response to TNF-α stimulation, creating a self-protective mechanism [47].

Proteomic Evidence:Compared with 2D monolayer culture, HeLa cells cultured in 3D exhibit significantly upregulated expression of endoplasmic reticulum (ER) stress-related proteins (e.g., HYOU1, HSPA5, HSP90B1), which enhance apoptosis resistance by maintaining ER homeostasis [22]. Aberrant expression of metabolism-related proteins such as voltage-dependent anion channel 1 (VDAC1) and hexokinase 2 (HK2) can influence cellular sensitivity to apoptosis inducers by regulating glucose metabolism [47].

Metabolomic Characteristics:High glycolytic activity and intracellular ATP accumulation provide survival advantages for cells in hypoxic microenvironments, while low tolerance to oxidative stress makes cells sensitive to ROS-induced mitochondrial damage [48]. Metabolite analysis shows that ROS accumulation leads to loss of mitochondrial membrane potential and ATP depletion, indicating a bidirectional regulatory relationship between metabolic reprogramming and apoptosis sensitivity [48].

4. AI modeling of the multi-pathway interaction network of HeLa cells

4.1. Comparison of artificial intelligence and traditional experimental methods in the study of HeLa cell pathways

In the study of pathways in HeLa cells, traditional experimental methods face multi-dimensional limitations. On one hand, conventional techniques such as flow cytometry, RT-qPCR, and MTT assay can only capture static and isolated molecular changes, such as specific cell cycle arrest or single-gene expression variations, and struggle to integrate multi-scale dynamic processes like metabolic reprogramming and crosstalk between signaling pathways [38,49]. Take single-cell lipid mass spectrometry imaging as an example: although this method can identify subcellular lipid

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heterogeneity, it cannot resolve the real-time interaction mechanisms between lipid metabolism and apoptotic pathways—a common limitation of traditional methods that severely restricts their application in dynamic analysis of cellular pathways [38]. On the other hand, traditional pathway construction relies on a hypothesis-driven "trial-and-error" model, requiring data alignment with existing pathways. However, due to the cancerous origin and long-term in vitro evolution of HeLa cells, many core pathways have undergone dysregulation, mutation, or reprogramming, potentially leading to atypical responses to stimuli and insufficient model predictability [49,50]. These limitations have severely hindered the development of precise intervention strategies targeting drug resistance and mutation-specific targets in HeLa cells.

Artificial intelligence (AI) technologies have significantly overcome these bottlenecks through multi-modal data integration and dynamic simulation, enabling successful integration of large-scale data and accurate prediction of cellular pathways. The deep learning model MoDL can segment mitochondrial morphology with high precision and predict functional states such as membrane potential and ROS generation, directly linking mitochondrial dynamics to the spatiotemporal evolution of apoptotic pathways [51]. The cross-species pre-trained model GeneCompass integrates 126 million single-cell datasets and fuses prior knowledge of gene regulation, significantly reducing errors (15.4%) and enabling accurate prediction of pathway responses to gene perturbations [52]. Additionally, AI-driven virtual cells (AIVC) can construct multi-scale neural networks to simulate HeLa cell behavior from molecular interactions to the tissue level, supporting "computational experiments" for optimizing drug combination design [53]. Furthermore, protein manipulation technologies like CAGE-Prox, combined with machine learning optimization, have achieved spatiotemporal regulation of tumor pyroptosis pathways in vivo, providing a "prediction-validation" closed-loop research platform for specific pathway intervention in HeLa cells [54,55]. These advancements mark the transition of HeLa cell research toward an intelligent research paradigm integrating "wet lab experiments" with "dry computational modeling.

4.2. AI reconstruction of cell communication networks

The cellular communication network serves as a core framework for understanding the interaction mechanisms within the microenvironment of HeLa cells and the signal transduction processes. AI-based algorithms can automatically construct multi-pathway interaction networks for HeLa cells by integrating known biological knowledge and experimental data, enabling cross-scale modeling from molecular interactions to systemic regulation.

In recent years, the introduction of deep learning technologies has provided entirely new tools for reconstructing high-precision cellular communication networks. In HeLa cell research, graph neural network (GNN)-based methods are particularly suitable for capturing complex intercellular signal transduction patterns. The DeepTFni algorithm, leveraging a graph neural network architecture, infers transcription factor (TF) regulatory networks from single-cell ATAC-seq data and demonstrates excellent performance even with limited cell numbers [56]. This technology can be applied to analyze communication networks in HeLa cells by integrating chromatin accessibility and gene expression data, thereby revealing dynamic communication patterns during cell state transitions. Additionally, physically inspired Transformer architectures represent a cutting-edge direction in cellular communication network modeling. The PISTE (Sliding-attention Transformer) algorithm incorporates physical priors to simulate gradient fields of biomolecular interactions, achieving high-precision prediction (accuracy >90%) of TCR-antigen-HLA binding [57]. This architecture can be adapted to study receptor-ligand interactions on the surface of HeLa cells, predict key molecular pairs involved in intercellular communication, and provide quantitative

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insights into the interactions between HeLa cells and immune cells. The AI-driven three-dimensional genomics technology DNAICI has opened new dimensions for studying communication networks in HeLa cells. Through multi-scale resolution adaptive analysis (50–500 kb), it integrates multi-omics data such as Hi-C and detects dynamic intra-chromosomal interactions using modularity optimization and the SLM algorithm. This technology has successfully identified 515 DIEGs in breast cancer cells (network stability RV=0.8), and its future application to HeLa cells may reveal the specific molecular mechanisms by which HPV-mediated three-dimensional chromatin reorganization influences gene expression related to cellular communication [58].

4.3. Modeling of cross-path interaction networks

The proliferation, migration, and malignant phenotype of HeLa cells often stem from the cooperative dysregulation of multiple signaling pathways. Modeling cross-pathway interaction networks is pivotal for understanding cervical cancer pathogenesis, and artificial intelligence (AI) technologies offer powerful tools to decipher such complex pathway interplays.

Multi-layered network integration has emerged as a key focus in current AI-driven signaling pathway research. DNAICI algorithm-identified DIEGs are significantly enriched in critical pathways like PI3K-Akt, which are also operational in HeLa cells. Applying this method to HeLa cells could therefore unveil interaction patterns among multiple oncogenic pathways—such as those reshaped by HPV oncoprotein-induced chromatin reorganization [58]. Drug perturbation response modeling employs machine learning/deep learning to predict cellular responses to drug interventions using transcriptome data [59,60]. A recent study on Alnus altaica's dichloromethane fraction combined flow cytometry, RT-qPCR, and AI analysis to reveal drug-induced coupling between cell cycle and apoptosis pathways [49]. The fraction induced HeLa cell apoptosis by upregulating Bax (2.96-fold) and p53 (2.60-fold) while downregulating Bcl-2 (0.6467-fold), exemplifying multi-gene cooperative regulation in pathway interactions.

The scNET deep learning framework, based on graph neural networks (GNNs), learns gene and cell embeddings by integrating single-cell RNA-seq (scRNA-seq) data with protein-protein interaction (PPI) networks [61]. Its dual-view architecture alternates propagation of gene expression and protein interaction networks, incorporating attention mechanisms to optimize cell-cell similarity graphs (KNN graphs) for noise reduction and condition-specific gene regulatory network capture [61]. An attention-based edge attention mechanism further refines these graphs to accurately capture dynamic intercellular relationships [61]. Applying scNET to HeLa cell pathway modeling enhances gene functional annotation and pathway feature identification, improving cell clustering and pathway analysis accuracy. This approach illuminates gene regulatory networks and pathway activation states under diverse conditions, offering novel insights for cancer research and therapy.

4.4. Drug target prediction and virtual screening

Drug development represents one of the core application directions in HeLa cell research, and artificial intelligence (AI) technologies are driving revolutionary transformations in anticancer drug target prediction and virtual screening workflows.

Binding affinity prediction based on physical mechanisms has become a focal point of current technological breakthroughs. The PISTE framework achieves precise prediction of TCR-antigen-HLA binding by simulating the gradient field of residue interactions, demonstrating a 75% clinical validation rate in prostate cancer research [57]. Similar principles can be applied to drug target prediction in HeLa cells, particularly for accelerating the design of novel protein degraders against

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traditionally "undruggable" protein-protein interaction (PPI) interfaces. Polypharmacological effect prediction is of particular importance for HeLa cell therapy. For example, the anticancer activity of the dichloromethane fraction from Alnus altaiensis stems from the synergistic induction of cell cycle arrest and apoptosis by terpenoids and fatty acids [49]. AI-powered systems pharmacology models can decipher such complex multi-target effects, predicting and optimizing the action mechanisms and combination ratios of natural extracts or compound formulations. Graph neural networks (GNNs) enable simultaneous modeling of multi-level relationships among compounds, targets, and pathways. By constructing deep learning-based dynamic models, AI technologies can simulate changes in intracellular pathway interactions under different conditions in HeLa cells and predict cellular responses to various stimuli [62]. Notably, the pathway interaction networks in HeLa cells undergo remodeling in disease states. AI can predict such network changes based on disease-related data, providing new targets and insights for disease diagnosis and treatment [3].

Geneformer, a deep learning model based on the attention mechanism, learns the dynamic changes of gene regulatory networks through pre-training on large-scale single-cell transcriptome data [63]. Utilizing self-supervised learning with a masked learning objective, the model gains a fundamental understanding of network dynamics during pre-training and encodes network hierarchies into its attention weights [63]. When applied to HeLa cell modeling, Geneformer requires only minimal fine-tuning with task-specific data to accurately predict gene dosage sensitivity, chromatin dynamics, and network hierarchies [63]. This feature enables Geneformer to more accurately identify key regulatory genes and pathways in HeLa cells, providing a powerful tool for understanding gene regulatory networks and discovering potential therapeutic targets.

5. Challenges and future directions

Single-cell sequencing and multi-omics technologies have enabled high-throughput analysis of HeLa cells (e.g., UDA-seq's 100,000+ cells/channel throughput [10]), but challenges remain in interpreting data due to genomic instability-driven subclonal diversity, dynamic pathway crosstalk, and epigenetic heterogeneity, while AI models like GLUE [25] and scGPT [5] lack experimental validation for HPV-related pathways and suffer from interpretability issues as "black boxes" [64]. Future directions include developing high-sensitivity spatial multi-omics technologies (e.g., UDA-seq + spatial transcriptomics [10,65]), constructing HeLa-specific AI models via transfer learning with causal inference modules (e.g., GLUE priors [25]), and establishing standardized datasets to validate 14+ prediction algorithms [2], which will advance cervical cancer research by revealing therapeutic targets and enabling personalized drug testing through organoid-single-cell platforms [66], ultimately shifting treatment toward predictive precision medicine.

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