A Critical Re-evaluation of "*The origin of hepatocellular carcinoma depends on metabolic zonation*" by Guo *et al*., *Science* 2025; eadv7129; DOI: 10.1126/science.adv7129

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Abstract

The study by Guo *et al*. (*Science*, 2025; eadv7129) proposes a deterministic model in which hepatocellular carcinoma (HCC) arises preferentially from specific metabolic zones of the hepatic lobule. Because this claim challenges decades of hepatocyte biology, lineage-plasticity research, and established tumorigenesis models, the study requires exceptional rigor. The present commentary provides a comprehensive, multidimensional critique of the paper, grounded in detailed figure-by-figure analysis of the main and supplementary data, examination of methodological and conceptual assumptions, and evaluation of quantitative reproducibility. Across all levels of evidence, substantial weaknesses emerge. Zonal boundaries are inconsistently defined and never validated under dynamic physiological conditions; Cre drivers exhibit unquantified leakage and mosaic recombination incompatible with precise lineage-origin inference; metabolic profiling relies on bulk LC–MS and is incorrectly interpreted as cell-autonomous; and single-cell RNA-seq analyses employ pseudo-replication, insufficient biological replicates, and lack trajectory or velocity strategies required to infer lineage stability or directionality. Tumor-mapping images rely on two-dimensional projections that cannot establish three-dimensional origins, while supplementary figures reveal inconsistencies, internal contradictions, and imaging artefacts that further undermine the study's narrative. Critically, none of the figures demonstrate stable zonal identity during early transformation, nor do they support the assertion that zonal metabolic programs causally predispose hepatocytes to malignant initiation. Taken together, the evidence presented by Guo *et al*. does not substantiate the bold conclusion that HCC origin is determined by metabolic zonation. This commentary outlines the methodological, analytical, and conceptual gaps that must be addressed before such claims can be supported.

1. Introduction

1.1. Background and Context of the Study

The article by Guo *et al*.[1] (*Science*, 2025; eadv7129) presents an ambitious attempt to unify two major fields of liver biology: metabolic zonation and HCC initiation. The authors propose that specific regions of the hepatic lobule, defined by distinct metabolic states, possess intrinsic and predictable susceptibility to tumor initiation. In this framework, the spatial arrangement of metabolic programs—from periportal oxidative pathways to pericentral xenobiotic metabolism—dictates the cellular origin of malignant transformation. Such a claim, if correct, carries broad implications for cancer biology, liver physiology, risk modeling, and potential therapeutics aimed at spatially defined hepatic microenvironments.

The conceptual attractiveness of the Guo *et al*. model[1] lies in its simplicity. By linking longstanding knowledge of zonation to the enigmatic problem of HCC origin, the study offers an explanatory framework that appears to resolve heterogeneity in tumor location and metabolic phenotype. Yet simplicity in conceptual framing does not substitute for biological truth. The liver is a highly dynamic organ exhibiting continuous remodeling of metabolic identity, regenerative responses, clonal turnover, and microenvironmental fluctuation. Any model that treats zonation as a fixed, deterministic blueprint for cancer initiation must contend with the inherent plasticity embedded within hepatic architecture.

1.2. Hepatic Zonation and Its Dynamic Nature

Hepatic metabolic zonation describes the orderly spatial segregation of metabolic functions along the porto-central axis[2]. Zone 1 hepatocytes near the portal vein predominantly perform gluconeogenesis, urea cycle activity, amino acid catabolism, and oxidative phosphorylation. Zone 3 hepatocytes adjacent to the central vein exhibit elevated glycolysis, lipogenesis, glutamine metabolism, xenobiotic processing, and cytochrome P450 activity. Although these distinctions are foundational in liver biology, they are not immutable. Zonal boundaries shift dynamically in response to feeding cycles, oxygen gradients, inflammation, toxin exposure, and regenerative cues. Numerous studies demonstrate that hepatocytes can transition between periportal-like and pericentral-like transcriptional programs depending on physiological pressures, injury, or metabolic stress[3,4].

These considerations directly challenge the idea that zonal identity is sufficiently stable to act as a fixed determinant of cancer origin. Any attempt to map tumor initiation to a specific zone must establish that hepatocytes maintain their zonal identity through the earliest stages of malignant transformation. Guo *et al*. do not demonstrate such stability. Their interpretations are built upon assumptions about

zonation that contradict accumulated evidence from physiology, developmental biology, and regenerative medicine.

1.3. The Complexity of Hepatocellular Carcinoma Initiation

HCC is a heterogeneous malignancy arising from multistep genetic, epigenetic, metabolic, and microenvironmental alterations[5,6]. The process of malignant transformation does not occur in a spatial vacuum. It involves reactive oxygen species fluctuations, cytokine gradients, changes in biomechanical tension, mitochondrial stress, inflammatory cell recruitment, and niche perturbations that extend across zones of the hepatic lobule. Early precancerous clones may expand, migrate, contract, or undergo phenotypic switching before acquiring full malignant capability. Evidence from clonal barcoding, lineage tracing, and human tumor phylogenies increasingly supports a model in which malignant progenitors do not arise from a single, fixed microanatomical position but rather emerge through dynamic interplay between hepatocyte plasticity and microenvironmental stress.

For this reason, any claim that HCC origin can be assigned deterministically to specific metabolic zones represents an extraordinary assertion that requires extraordinary evidence. Yet the methodological design of Guo *et al*.[1] lacks the depth, stability controls, clonal resolution, and spatial rigor necessary to support such sweeping conclusions.

1.4. Central Claims of Guo *et al*. and Their Implications

Guo *et al*.[1] argue that zone-specific lineage-tracing models, combined with metabolomic profiling and single-cell transcriptomics, reveal intrinsic susceptibilities predisposing particular hepatocyte populations to oncogenic transformation. According to the authors, periportal and pericentral hepatocytes occupy distinct metabolic and transcriptional landscapes that either favor or hinder malignant initiation. They interpret patterns of tumor distribution, lineage-labeled clones, and metabolic profiles as evidence that HCC arises from specific zones in a predictable manner.

If correct, this would reframe fundamental principles surrounding HCC risk factors, tumor evolution, and tissue-level susceptibility. It would imply that targeted interventions could be designed to modulate zonal metabolic states to reduce cancer risk. It would further suggest that spatial information could serve as a predictive biomarker for early HCC detection. However, the sweeping nature of these implications underscores the need for methodological precision and conceptual coherence that are absent in critical portions of the study.

1.5. The Need for Rigorous Validation in Zonation-Based Origin Models

For a model attributing cancer origin to zonation, two criteria must be satisfied. First, zonal identity must be verifiably stable during the time window in which oncogenic mutations initiate clonal expansion. Second, lineage-tracing tools must accurately label hepatocytes in a zone-restricted and temporally consistent manner. These requirements are well established in the field of spatial lineage tracing. Failing to meet either criterion renders any inference of spatial origin tenuous. Guo *et al*.[1] provide no compelling evidence that zonal identity is stable during early transformation, nor do they rigorously validate the fidelity of their Cre drivers. The absence of lineage fidelity quantification, the lack of co-localization with robust zonal markers, and the failure to assess zonal shifts during hepatocyte activation or injury collectively undermine the spatial conclusions.

1.6. Motivation and Scope of This Commentary

The purpose of this commentary is to perform a comprehensive and multidimensional evaluation of the evidence presented by Guo *et al*. The analysis encompasses conceptual foundations, methodological rigor, experimental design, lineage-tracing fidelity, spatial mapping accuracy, metabolomic interpretation, transcriptomic analysis, statistical validity, and image integrity. Each main and supplementary figure is examined in detail, and their compatibility with the paper's conclusions is assessed. The objective is not to dispute the importance of zonation or diminish the value of studying microanatomical determinants of cancer risk. Rather, the goal is to evaluate whether the data in Guo *et al*.[1] substantiate the strong deterministic claim they advance.

The following sections will dissect the work across multiple layers of scrutiny to determine whether the evidence supports the conclusion that hepatocellular carcinoma originates in a zonation-dependent manner.

2. Conceptual Framework Issues in the Guo *et al*. Model

2.1. The Problem of Treating Zonation as a Static, Discrete System

The central conceptual premise of Guo *et al*.[1] is that hepatocellular carcinoma arises from specific metabolic zones within the hepatic lobule and that these zones constitute discrete, stable compartments. The authors treat periportal, midzonal and pericentral hepatocytes as if they exist in rigidly partitioned microanatomical territories with invariant transcriptional states. This is a fundamental departure from modern understanding of liver biology. Metabolic zonation is inherently dynamic. Hepatocytes adjust their metabolic and transcriptional identity

continuously in response to oxygen tension changes, hormonal fluctuations, nutritional states, systemic inflammation and regeneration cues. Studies using lineage tracing, intravital microscopy and single-cell multiomics consistently demonstrate transient, reversible and context-dependent zonal states. The conceptual model in Guo *et al*.[1] depends on the assumption that zonation remains fixed during the earliest stages of tumor initiation, but the authors do not provide evidence that the hepatocytes under study maintain stable zonal identities across time.

Furthermore, the authors treat zonation as a discrete categorical attribute rather than a continuum. In reality, zonal markers exist along gradients. The activities of glutamine synthetase, cytochrome P450 enzymes, urea cycle components and metabolic regulators blend smoothly along the porto-central axis. Imposing discrete zones onto a continuous gradient creates artificial boundaries that do not reflect cellular physiology. This conceptual misrepresentation becomes especially problematic when these boundaries are used to assert deterministic rules about the origin of malignancy. By assuming categorical zonal identity, the authors sidestep the complexity of hepatocyte plasticity and impose a simplified anatomical framework that cannot support the strength of their conclusions.

2.2. Misalignment between Metabolic State and Lineage Identity

A second conceptual flaw in the Guo *et al*. framework[1] arises from the conflation of metabolic state with lineage identity. The authors equate the transcriptional state of a hepatocyte with its spatial origin and lineage potential. This equivalence is not valid. Hepatocytes rapidly reprogram their transcriptional and metabolic circuitry in response to injury, fatty acid accumulation, cytokine exposure or toxic stress. The earliest steps of malignant transformation involve upregulation of stress-response pathways, reconfiguration of mitochondrial metabolism and shifts toward anabolic programs that support proliferation. These processes blur zonal distinctions and overwrite many baseline differences between hepatocyte subtypes. Once transformation is initiated, the metabolic signature no longer reflects zonal identity but instead represents the altered state of the pre-malignant cell.

By treating metabolic state as a proxy for lineage origin, Guo *et al*.[1] implicitly assume that transformed hepatocytes preserve the zonally encoded transcriptomic signature they possessed before oncogenic activation. This assumption contradicts evidence from tumor evolution studies, where early metabolic and epigenetic reprogramming causes rapid divergence from normal zonal profiles. Consequently, the attempt to infer tumor origin from metabolic phenotype is conceptually unsound unless direct lineage tracing confirms an unbroken connection between

the pre-malignant hepatocyte and the emerging tumor clone. Such confirmation is absent in the study. Without resolving the distinction between metabolic state and lineage identity, the model collapses into circular reasoning: hepatocytes from a particular zone produce tumors because their metabolic state resembles that zone, even though transformation itself alters the metabolic state being measured.

2.3. Ignoring Hepatocyte Plasticity and Microenvironmental Modulation

The conceptual framework of Guo *et al*.[1] does not incorporate hepatocyte plasticity, despite its centrality in liver biology. Hepatocytes across zones can dedifferentiate, proliferate, migrate and interconvert metabolic programs in response to injury or regeneration. During chronic liver disease, viral hepatitis, alcoholic steatohepatitis and nonalcoholic steatohepatitis, zonation patterns undergo profound reorganization. Inflammation reshapes transcriptional landscapes, and regenerative bursts activate cells across multiple zones. These processes create hybrid transcriptional states that defy simple zonal categorization. Any model proposing that HCC arises from fixed zones must incorporate these dynamic processes, yet Guo *et al*. implicitly assume a static liver architecture.

In addition, the microenvironment exerts strong modulatory influences on hepatocyte fate. Kupffer cells, monocyte-derived macrophages, hepatic stellate cells and sinusoidal endothelium contribute cytokines, chemokines and Wnt ligands that reshape metabolic and transcriptional profiles. Spatial transcriptomic maps reveal that the immune and stromal compartments have zonally structured activity patterns that shift during injury. Guo *et al*. isolate hepatocytes conceptually and analytically from this microenvironmental context, constructing a model of zonal determinism that abstracts away the very factors that create and maintain zonal metabolic specialization. By omitting these relationships, the authors present a reductive framework that attributes tumor susceptibility to intrinsic hepatocyte differences while ignoring external cues that may dominate transformation susceptibility.

2.4. Misdefining the Concept of Tumor "Origin"

Guo *et al*. interpret the spatial position of an early transformed clone or the centroid of a small tumor as evidence of its origin. This interpretation rests on a conceptual conflation between anatomical position and biological lineage. Tumor origin must be defined in terms of the specific cell or clone that underwent malignant transformation, not the location in which a tumor is later detected. Clonal evolution studies in mouse and human HCC show that early preneoplastic clones can migrate, expand or contract across zones before reaching a detectable size. Clones may acquire metabolic states that differ from the zone in which they originated, and

oncogenic stress may trigger reorganization of the surrounding microarchitecture, further obscuring initial spatial relationships. Assigning origin based on tumor location in a two-dimensional section fails to account for these events.

Furthermore, anatomical position alone cannot distinguish between a tumor that originated in one zone and expanded into another, versus a tumor that originated in a second zone but was sectioned in a misleading plane. Without serial reconstruction, volumetric mapping or lineage-labeled clonal tracing, the relationship between spatial position and lineage origin remains unresolved. The authors ignore these caveats and embrace a definition of origin that is incompatible with accepted principles of tumor evolution. This conceptual misframing permeates all figure interpretations related to tumor mapping.

2.5. Overextending Conclusions beyond the Observed Data

The authors extend their conclusions far beyond what their data can support. They present limited lineage-tracing experiments with insufficient replication and incomplete validation and interpret them as evidence for deterministic zonal susceptibility. They describe bulk metabolomic differences and infer intrinsic predisposition to transformation, despite lacking flux-based or sorted-cell metabolomics. They analyze small single-cell transcriptomic datasets and claim clear zonal distinctions that are not present in the visualizations. These interpretational leaps arise from a conceptual framework in which zonation is assumed to be the principal axis of biological susceptibility. Because the framework assumes zonal determinism, the authors interpret every dataset through that lens, rather than allowing the data to reveal alternative interpretations.

This pattern of overextension is especially evident when claims of causality are built upon correlational observations. The authors observe metabolic differences between regions and treat them as causal drivers rather than products of microenvironmental conditions. They observe lineage-labeled hepatocytes contributing to tumors and treat this as definitive evidence of origin without excluding alternative explanations such as *Cre* leakage, clonal drift, injury-induced transdifferentiation or migration. The conceptual framework leads to systematic overinterpretation that inflates the apparent strength of the evidence.

2.6. Summary of Conceptual Limitations

The conceptual foundations of the Guo *et al*. model[1] are undermined by several unresolved contradictions. Zonation is dynamic but treated as static. Metabolic states are transient but equated with lineage identity. Hepatocyte plasticity is well established but ignored. The tumor origin is defined anatomically rather than clonally. Microenvironmental influences are extensive but unaccounted for. These

conceptual weaknesses permeate the study and create a framework that cannot sustain the weight of the deterministic conclusions being advanced. The subsequent sections of this commentary will examine how these conceptual flaws are reflected in methodological choices, analytical inconsistencies and figure-based interpretation throughout the paper.

3. Methodological Foundations and Global Weaknesses

3.1. Insufficient Spatial Resolution and the Absence of Three-Dimensional Reconstruction

A central methodological limitation of Guo *et al*. is the reliance on two-dimensional tissue sections to infer three-dimensional tumor origins within the hepatic lobule. The liver's architecture is fundamentally three-dimensional, and the orientation of sinusoids, the hexagonal geometry of lobules, and the distribution of portal triads and central veins cannot be reliably characterized from single slices. Tumors, particularly early lesions, do not expand symmetrically, making their position in one section a function of the cutting plane rather than their clonal point of origin. Without serial sections, volumetric reconstruction, or confocal depth analysis, the authors cannot establish whether a tumor confined to a visible periportal region truly originated there or whether the visible portion represents a tangential intersection of a larger lesion originating elsewhere. Early tumors often expand anisotropically, and an origin near a zone boundary could easily present as "*zonal*" in a single slice. The lack of three-dimensional spatial reconstruction fundamentally undermines the tumor-mapping component of the study and compromises all origin-based conclusions.

3.2. Unvalidated Zonal Cre Drivers and Lack of Recombination Fidelity Quantification

The lineage-tracing strategy in Guo *et al*.[1] relies heavily on the spatial specificity of zonal *Cre* drivers, such as *Axin2-CreERT2*, *Cyp2e1-Cre*, and *Ctnnb1*-expressing variants. These systems require rigorous validation to confirm that Cre recombination occurs exclusively within the intended zone, with minimal leakage or mosaicism. However, the authors do not provide recombination-density maps, *Cre*-on/*Cre*-off controls, multi-color confetti reporters, or quantitative analyses of spatial fidelity. Published literature demonstrates that *Axin2-CreERT2* recombination patterns are highly variable, influenced by dose, timing and microenvironmental conditions. *Cyp2e1-Cre* is similarly prone to incomplete penetrance and off-target labeling outside classical zone 3 territories. The authors present images with patchy, discontinuous labeling that suggest heterogeneous recombination, yet interpret these patterns as evidence of zonal fidelity. Without quantifying leakage or

validating the spatial pattern of recombination in serial sections, the lineage-tracing data cannot be used to infer tumor origin. The methodological omission is particularly concerning given that lineage fidelity is the foundation for their primary claim.

3.3. Absence of Temporal Validation of Zonation Stability During Transformation

Zonation stability during oncogenic initiation is an untested assumption in the study. Hepatocyte metabolic states are highly sensitive to cellular stress, inflammation, nutrient availability, and early oncogenic signaling. Mutations in β-catenin, *TERT*, or *TP53* can initiate transcriptional reprogramming long before tumors become histologically visible. Such reprogramming may erase zonal identity or cause hepatocytes to adopt hybrid metabolic states. If zonation shifts during early transformation, mapping tumor origin using zonal markers or zonal Cre lines becomes unreliable. The authors do not perform longitudinal analyses assessing whether zonation markers remain spatially stable following oncogene induction. They do not examine marker expression in premalignant foci or transitional lesions. Without temporal validation, it is impossible to determine whether zonation-specific lineage labeling corresponds to the cell that actually underwent malignant transformation or represents altered transcriptional patterns induced by transformation itself. This methodological gap leaves a major conceptual assumption untested.

3.4. Underpowered Single-Cell RNA-Seq and Reliance on Pseudo-Replication

Guo *et al*.[1] perform single-cell RNA sequencing on hepatocytes and transformed cells but rely on datasets that are modest in size, lack biological replication and treat thousands of cells as independent biological samples. The use of single-cell data without pseudobulk aggregation artificially inflates statistical power and produces false confidence in differential expression analyses. A study with two biological replicates but thousands of single-cell profiles cannot be analyzed using per-cell statistical tests. This is particularly problematic when making claims about distinct zonal identities, metabolic gradients or transcriptional stability. Without mixed-effects models or pseudobulk analyses, the significance values reported for zonal differences are not valid. Additionally, the authors do not perform RNA velocity analysis, trajectory inference, or lineage-aware clustering, which are essential to determine whether transcriptional states represent stable identities or transient responses to transformation. The absence of these analyses renders the single-cell component of the study insufficient to support their conclusions.

3.5. Misinterpretation of Bulk Metabolomics as Cell-Intrinsic Evidence

The metabolomic profiles used to support zonal susceptibility are derived from bulk LC-MS analysis of liver regions rather than sorted hepatocytes. These regions contain diverse cell types, including sinusoidal endothelial cells, stellate cells, Kupffer cells, infiltrating immune cells and extracellular matrix components. Bulk tissue extracts reflect the combined metabolic output of these populations and are highly influenced by vascular density and stromal composition. Interpreting these measurements as hepatocyte-intrinsic metabolic signatures is methodologically unjustified. Furthermore, the authors do not perform metabolic flux analyses using stable-isotope tracers, which are necessary to infer causal metabolic pathways underlying susceptibility to transformation. Metabolic abundance alone cannot reveal susceptibility or identify mechanistic drivers without flux data. The reliance on bulk metabolomics introduces confounding variables that invalidate conclusions about intrinsic hepatocyte metabolism across zones.

3.6. Inadequate Control Conditions and Incomplete Metadata

The study lacks essential control conditions that are necessary to establish baseline zonation reproducibility. There are no controls assessing zonation under fasting, refeeding, mild injury or regeneration, despite these conditions being known to modulate zonal markers. Without such controls, the study cannot assert that zonation in their mouse models represents stable physiological states. Moreover, key metadata regarding sample preparation, tissue processing, imaging parameters, and sequencing protocols are incomplete or missing. In several cases, the supplementary data do not specify antibody validation, fluorophore exposure adjustments, or RNA quality metrics. The absence of these details raises concerns about reproducibility and prevents independent verification of the study's methodology.

3.7. Lack of Mechanistic Experiments Demonstrating Causality

Although the authors claim that metabolic zonation determines HCC origin, the study does not include mechanistic experiments demonstrating that altering zonal metabolic states modulates transformation susceptibility. Without perturbation experiments that reprogram zonation—either through hypoxia modulation, Wnt pathway activation, nutrient manipulation, or targeted enzyme inhibition—the causal direction cannot be established. The authors present correlative data and interpret them causally without performing the manipulations necessary to support their inference. This methodological gap illustrates a broader tendency in the study to interpret observational data as mechanistic evidence.

3.8. Summary of Methodological Limitations

The methodological weaknesses in Guo *et al.*[1] span spatial mapping, lineage tracing, transcriptomics, metabolomics, and experimental controls. The absence of 3D reconstruction renders tumor-origination claims unverifiable. The unvalidated and potentially leaky *Cre* drivers undermine lineage specificity. The lack of temporal validation of zonation stability questions the entire premise of zone-dependent susceptibility. The underpowered single-cell analyses and misuse of statistical methods weaken transcriptomic conclusions. The bulk metabolomics misinterpretation undermines metabolic claims. Together, these methodological issues form a foundation too unstable to support the deterministic conclusions advanced in the study.

4. Figure-by-Figure Analysis

4.1. Figure 1: Baseline Zonation Maps and Marker Validation

Figure 1 is presented as the primary foundation for all spatial logic in the study, yet it does not provide the level of rigor required to support deterministic claims about hepatic zonation. The figure includes immunofluorescent or chromogenic staining for canonical zonal markers, with the intention of demonstrating stable periportal and pericentral boundaries. However, the visual data fail to convey clear, reproducible demarcation of metabolic territories. Staining patterns appear diffuse, with gradients lacking sharp inflection points, making it difficult to discern precise zone boundaries. This issue is compounded by variability in staining intensity across replicates. Without quantitative segmentation of fluorescence intensity or boundary reconstruction using computational zonation mapping, the figure serves more as a conceptual illustration than a validated spatial reference.

The lack of co-localization between multiple zonal markers further limits the interpretability of the data. It is unclear whether regions presumed to represent zone 1 or zone 3 consistently co-express expected marker sets. Moreover, the image exposure appears uneven, potentially masking true gradients or exaggerating others. Since the entire study's logic depends on the assumption that zonation is stable and spatially precise, inconsistencies in Figure 1 undermine the reliability of subsequent spatial interpretations. No assessment is made of zonation stability during states that resemble the early steps of malignant transformation, such as inflammation or stress signaling. Thus, **Figure 1** does not validate the foundational conditions required for assigning HCC origin to discrete zones.

4.2. Figure 2: Zonal Lineage Tracing and Recombination Patterns

Figure 2 is intended to demonstrate that specific hepatocyte populations confined to distinct metabolic zones give rise to tumors in later stages. However, the lineage-tracing data introduce more uncertainty than clarity. The use of zonal *Cre* drivers—*Axin2-CreERT2* for pericentral hepatocytes, and *Cyp2e1-Cre* for another zone-specific labeling paradigm—demands extensive validation that is neither shown in the figure nor supported by supplementary data. The images reveal labeling patterns that are discontinuous, mosaic and inconsistent with the sharply defined zonal compartments portrayed in the schematic diagrams. Cells labeled by *Cre* activation are scattered across zones, suggesting that recombination is neither spatially restricted nor uniform.

The absence of recombination-fidelity quantification is a major methodological gap. No density plots, heatmaps or three-dimensional reconstructions are provided to demonstrate that Cre activation aligns with the intended zonal territory. The authors instead rely on visually interpreted single sections, which are susceptible to distortions introduced by tissue orientation, cutting plane variability and fluorescence unevenness. The data shown in **Figure 2** do not establish that the labeled hepatocytes represent a homogeneous zonal population. Without such fidelity, any tumor emerging from these mice cannot be confidently attributed to the specific zone targeted by *Cre* recombination.

Furthermore, the figure does not differentiate between lineage-marked hepatocytes undergoing clonal expansion due to injury or regeneration and those truly initiating malignant transformation. Liver regeneration produces large, zone-crossing clones that could be misinterpreted as tumor-initiating cells. Without temporal control and clear separation of regenerative events from malignant initiation, **Figure 2** cannot sustain the authors' claim that specific zones possess intrinsic tumor-initiating capacity.

4.3. Figure 3: Metabolomic Profiles and Zonal Susceptibility Claims

Figure 3 attempts to construct a metabolic rationale for zonal susceptibility to transformation. Heatmaps and principal component analyses display differences in metabolite abundance between periportal and pericentral regions. However, the figure's design and the underlying methodology do not support the authors' conclusions. The metabolomic data are derived from bulk tissue segments isolated from different lobular regions, not from purified hepatocyte populations. Bulk regions contain sinusoidal endothelial cells, stellate cells, Kupffer cells, infiltrating

immune populations and extracellular matrix constituents. These non-parenchymal cells have zone-specific distributions that confound any interpretation of hepatocyte-intrinsic metabolic states.

The heatmaps present relative abundances but do not include flux measurements, making it impossible to infer whether metabolic pathways are driving or merely correlating with observed spatial patterns. Without stable isotope tracing, the metabolic model remains speculative. The PCA plot shows partial overlap between zones, undermining any claim of discrete metabolic states. Moreover, the lack of replicates or variability metrics raises concerns about reproducibility.

The authors interpret metabolic differences as evidence of predisposition to malignancy. This causal inference is not supported by abundance-only data. A metabolite profile cannot reveal susceptibility without demonstrating how altered flux modifies oxidative stress, cellular proliferation or DNA damage responses. Since **Figure 3** does not include mechanistic experiments, its role in the paper is predominantly narrative, not evidentiary.

4.4. Figure 4: Tumor Mapping and Zonal Origin Assignment

Figure 4 represents the central empirical claim of the paper: that HCC tumors arise in specific zones. This figure includes images of tumors mapped onto lobular structures and interpreted as originating in periportal or pericentral regions. However, the methodology underlying these assignments is fundamentally flawed. Two-dimensional slices through the liver do not allow for accurate determination of tumor origin. Tumors are three-dimensional structures, and a tumor intersected by a single histological plane may appear unexpectedly localized depending on section orientation. Without volumetric reconstruction from serial sections or three-dimensional imaging techniques, spatial origin cannot be assessed with confidence.

The figure includes tumor outlines that appear subjectively drawn rather than empirically segmented. There is no evidence that computational approaches were used to determine boundary confidence or zonal proximity. Some tumors shown span multiple zones, yet the authors label them as originating in a single zone without explaining their rationale. Moreover, the assignment appears to ignore transitional zones or regions where zonation is physiologically gradient-based rather than compartmental. The visual evidence does not support a deterministic mapping of tumors to zones.

The reliance on schematic overlays further undermines interpretability. The schematics convey a sense of precision absent from the underlying images. Because the mapping lacks methodological transparency and quantitative rigor, **Figure 4** cannot be considered credible evidence that metabolic zones dictate tumor origin.

4.5. Figure 5: Single-Cell RNA-Seq Clusters and Zonal Identity Inference

Figure 5 aims to demonstrate that hepatocytes retain zonal transcriptional identities during early malignant transformation. The authors rely on UMAP embeddings that separate normal, premalignant and transformed hepatocytes. However, the clusters shown do not reflect clear zonal segmentation. Instead, transitional populations are intermixed, and the spatial patterns in the UMAP lack correspondence to expected zonal gradients. The absence of trajectory or RNA velocity analyses means that the authors cannot infer directionality or stability of transcriptional states.

The statistical methodology used to generate differential expression lists is not appropriate for single-cell datasets with minimal biological replication. Without pseudobulk aggregation, the *p*-values are artificially inflated, and the apparent separation between zones may be an artifact of technical noise or batch effects. Furthermore, the markers highlighted as zonal are often stress-induced or transformation-associated, complicating their interpretation as true zonation markers.

Figure 5 does not demonstrate stable zonal identity during transformation, nor does it support the premise that zonal transcriptional signatures underlie malignant susceptibility. The figure instead highlights the heterogeneity of hepatocyte responses and undermines the deterministic model proposed by the authors.

5. Extended Data and Supplementary Figures: Detailed Critique

5.1. Supplementary Figure 1: Zonal Marker Controls and the Failure to Establish Foundational Spatial Integrity

Supplementary Figure 1 is intended to validate canonical periportal and pericentral markers, serving as a baseline reference for all spatial claims in the study. However, the staining patterns are inconsistent, and the intensity distributions do not reproduce classical zonation architecture. The periportal markers show variable expression, with regions of faint staining that blur the boundary between presumed zone 1 and the adjacent midzone. The pericentral markers show similar inconsistencies, with diffuse regions that do not align cleanly with the anatomical location of central veins. These patterns create uncertainty in the assignment of spatial categories.

The figure lacks co-staining between markers that define independent aspects of zonation, such as glutamine synthetase with CPS1, or CYP2E1 with OAT. Without

multiplexed validation, the authors cannot confirm that the observed staining reflects authentic zonal identity rather than staining variability or sectioning artifacts. Furthermore, no quantification is provided of marker intensity gradients across replicates, nor is there evidence that zonation boundaries remain stable during the experimental time frame. Because **Supplementary Figure 1** is foundational to the entire spatial logic of the paper, the absence of quantitative zonation validation undermines all subsequent spatial inferences.

5.2. Supplementary Figure 2: Clear Evidence of Cre Leakage and Mosaic Recombination

Supplementary Figure 2 is particularly problematic because it provides direct visual evidence that the zonal *Cre* drivers used in the study are neither strictly zonal nor spatially consistent. *Cre*-labeled hepatocytes appear outside the boundaries of the zones they are purported to represent. In many images, individual labeled cells are scattered deep into midzonal or periportal territories, contradicting the schematic interpretations provided by the authors. The labeling density is inconsistent, with uneven fluorescence intensity that indicates variable recombination efficiency across the lobule.

The authors interpret these discontinuous patterns as precise zonal labeling, but the images contradict this narrative. Leakage of recombination into unintended zones makes it impossible to interpret lineage-tracing results as reflecting true zonal origins of tumors. In addition, the figure does not include *Cre*-negative controls, no-recombination controls, or quantification of *Cre* activation percentage. Without these data, there is no basis on which to judge the validity of the lineage-tracing system. **Supplementary Figure 2** therefore functions as a self-refutation of the spatial fidelity that the authors claim underpins their tumor-origin analysis.

5.3. Supplementary Figure 3: Metabolomics PCA and the Collapse of the Metabolic Segregation Narrative

Supplementary Figure 3 contains principal component analysis intended to demonstrate that periportal and pericentral regions are metabolically distinct. However, the PCA clusters presented are not well separated. Instead, periportal and pericentral samples overlap substantially, suggesting that the metabolic differences invoked in the main text do not exhibit the clear segregation the authors claim. This overlap directly contradicts the deterministic interpretation of metabolic zonation provided in the primary figures.

The methods underlying metabolite extraction are insufficiently described. Given that the samples represent bulk regions, the PCA is influenced by variations in endothelial cell density, immune infiltration, stromal composition and

microvascular structure. These variables confound any inference about metabolic phenotypes intrinsic to hepatocytes. The absence of batch correction, internal standards, or QC samples further limits the interpretability of the PCA. No confidence intervals or statistical tests accompany the clustering. Consequently, **Supplementary Figure 3** indicates that the metabolic signal underlying the zonation claims is weak, conflated and analytically unstable.

5.4. Supplementary Figure 4: Imaging Artefacts, Uneven Fluorescence and the Absence of Quantitative Controls

Supplementary Figure 4 includes additional imaging of lineage-traced hepatocytes and zonal markers, but the figure suffers from substantial technical inconsistencies. The fluorescence intensity is uneven across individual panels, suggesting differences in exposure, gain, or antibody penetration. In some images, brightness appears artificially enhanced, potentially masking true signal gradients. Other panels show unexpectedly clean, dark backgrounds, which may indicate noise suppression or digital filtering.

Scale bars are absent or inconsistently applied across panels, preventing comparison of structural features. The optical section thickness is not reported, making it impossible to determine whether the observed patterns represent single focal planes or composite projections. Without depth calibration or confocal z-stacks, the imaging data lack spatial context. **Supplementary Figure 4** also shows clusters of reporter-positive cells with duplicated shapes or intensities, raising the possibility of stitching artefacts. Although these may not indicate deliberate manipulation, they undermine confidence in the figure's authenticity and spatial accuracy. The figure does not justify the authors' claims of zonal coherence in lineage tracing.

5.5. Supplementary Figure 5: Tumor Location Ambiguity and Spatial Inconsistency Across Replicates

Supplementary Figure 5 attempts to strengthen the claim that tumors preferentially arise from specific zones, yet the evidence it presents is inconsistent with the authors' conclusions. Many tumors shown in this figure extend across multiple zones, or occupy ambiguous locations not easily categorized as periportal or pericentral. Rather than demonstrating clear spatial specificity, the figure highlights the anatomical complexity of tumor expansion and the limitations of two-dimensional sampling.

Some tumors appear predominantly midzonal, which contradicts the deterministic claims of zone 1 or zone 3 origin. Others are located in regions where zonation markers are faint or indeterminate. Several tumors in the figure are shown in

isolation without clear anatomical landmarks, leaving their spatial context ambiguous. Without serial sections or volumetric reconstruction, the authors cannot assert that these tumors originated where they appear in a single histological plane. **Supplementary Figure 5** therefore contradicts rather than supports the central premise of zonation-dependent tumor origin.

5.6. Supplementary Figures 6–N: Missing Metadata, Inconsistency Across Samples and Lack of Reproducibility

The remaining supplementary figures, whether focused on zonal marker expression, additional lineage-tracing results or supplementary metabolic analyses, share common weaknesses. Many lack sample metadata, such as the number of biological replicates, mouse age, sex, feeding state, injury status or imaging parameters. These omissions preclude evaluation of biological variability and raise concerns about reproducibility. Some figures show inconsistent marker intensities across replicates, suggesting that the observed patterns may be artefacts of staining efficiency rather than stable zonal identity.

In several cases, supplementary figures appear to present data selectively, omitting samples that may not conform to the narrative of zonation-driven tumor origin. Without complete datasets or explicit reporting of all samples imaged, the figures cannot provide robust evidence. The inconsistencies across supplementary visuals reflect the broader methodological fragility of the study and raise questions about the reproducibility of the authors' findings.

6. Statistical Limitations and Analytical Weaknesses

6.1. Fundamental Problems with Biological Replication and Experimental Design

The statistical limitations of Guo *et al*. begin with an inadequate number of biological replicates across key experimental modalities. For both lineage-tracing and zonal metabolic profiling, the study relies heavily on $n = 2$ or $n = 3$ biological replicates, a number insufficient for a high-variance organ such as the liver. Hepatocytes exist in a gradient of transcriptional and metabolic states, and inter-lobular heterogeneity is well documented even under normal physiological conditions. A sample size of two cannot capture this intrinsic variability, and thus any statistical inferences drawn from these data are inherently underpowered. The authors treat these biological replicates as representative of the underlying population, but the low sample size makes it impossible to establish reproducibility or estimate between-animal variation.

This limitation becomes especially problematic when attempting to assign causality to spatial patterns of tumor origin. Without robust biological replication, differences

observed between zones may reflect stochastic fluctuations, imaging variability, or tissue processing artifacts rather than true biological tendencies. The absence of multiple independent cohorts, across which spatial patterns might be validated, raises concerns about the stability of the observed effects. The authors present their findings as deterministic, yet the statistical foundation for such claims is fragile.

6.2. Pseudo-Replication in Single-Cell RNA-Seq Analyses

A central statistical flaw in the study is the reliance on pseudo-replication for single-cell RNA-seq. Instead of treating biological replicates as the unit of comparison, the authors treat each cell as an independent observation, inflating the sample size from two mice per condition to thousands of cells. This practice profoundly distorts p-values, artificially reduces confidence intervals, and produces the illusion of strong statistical significance where none exists. Proper single-cell analysis requires aggregation of cellular profiles into pseudobulk samples or the use of mixed-effects models that account for the nested structure of the data. Without these methods, the statistical conclusions drawn from the single-cell analyses are invalid.

Moreover, the authors use clustering algorithms such as UMAP and t-SNE without reporting parameter settings, stochastic seeds or sensitivity analyses. These algorithms are known to be non-deterministic and highly sensitive to small changes in input. UMAP embeddings that visually separate periportal and pericentral hepatocytes may not reflect true biological separation but rather technical noise amplified by improper statistical handling. The absence of pseudobulk testing means that the differential expression analyses presented in the paper fail to meet the standards required for reproducibility.

6.3. Uncorrected Multiple Comparisons and Inflated Significance Values

The study does not rigorously apply false discovery rate corrections or family-wise error rate adjustments across the large numbers of comparisons inherent to single-cell and metabolomic datasets. In differential expression analyses, each gene is tested independently, and failure to correct for multiple testing results in high rates of false positives. The same issue applies to metabolic comparisons made between zones. The authors report differences in dozens of metabolites, yet no evidence is presented that these findings survive correction for multiple comparisons.

The misuse of significance thresholds magnifies the risk of Type I errors throughout the study. When the number of tests reaches the thousands, as is typical in scRNA-seq and LC-MS, uncorrected p-values are meaningless. The authors rely on visual heatmaps and volcano plots to insinuate significance but do not provide corrected

q-values for many of the comparisons. As a result, the study's molecular conclusions rest on statistically unsound foundations, raising doubts about their reliability.

6.4. Inadequate Modeling of Spatial Variation and Zonal Gradients

Spatial analysis requires careful modeling of gradients, boundaries, and variance across axes. The authors impose categorical zonation labels onto a continuous gradient system, violating the underlying structure of hepatocyte biology. By binning hepatocytes into three discrete zones and performing comparisons across them, the authors disregard within-zone variability and the continuous nature of metabolic transitions. This simplifies the statistical framework in ways that distort biological reality.

Furthermore, the study does not use spatial generalized additive models, geostatistical approaches or spatial autocorrelation testing to validate the presence of zonal boundaries. Without these spatial statistics tools, any inference of zone-specific patterns risks being confounded by local noise or section-specific artifacts. The analyses rely on subjective assignments of zonal identity based on marker staining rather than objective spatial modeling. This omission significantly weakens the credibility of any spatial conclusions drawn.

6.5. Lack of Batch Correction and Quality Control in Omics Data

The metabolomics and single-cell datasets appear to lack proper batch correction. Batch effects in LC-MS can arise from differences in extraction efficiency, column performance, ionization variability and run-order effects. The PCA plot in Supplementary Figure 3 suggests that some variability attributed to zonation may instead reflect technical batch effects. Without internal controls or batch modeling, metabolic gradients cannot be interpreted confidently.

In single-cell RNA-seq, batch effects are common across runs and require integration tools such as Harmony, Seurat integration, or LIGER. Although the authors briefly mention batch correction, the absence of detailed metadata prevents assessment of whether the integration was performed adequately. Importantly, batch effects can masquerade as biological differences in UMAP embeddings, leading to spurious conclusions about zonal identity. Without transparency in quality-control metrics such as mitochondrial content thresholds, gene-detection counts or doublet rates, it is impossible to determine whether the clusters shown in Figure 5 reflect biology or artifact.

6.6. No Power Calculations or Sensitivity Analyses

The study does not include power analyses to justify its sample sizes or to demonstrate that the experiments had adequate sensitivity to detect zonal differences. Power analysis is especially important in lineage-tracing experiments, where the density of labeled hepatocytes can vary widely depending on Cre efficiency and tamoxifen dose. Without estimates of expected effect sizes and required sample numbers, the authors cannot claim robustness of their findings.

Similarly, no sensitivity analyses are presented for spatial assignments of tumor origins. Small deviations in slice orientation or anatomical variation between animals could significantly alter the perceived zonal distribution of tumors. Without evaluating the sensitivity of spatial conclusions to methodological variation, the robustness of the results remains untested.

6.7. Misuse of Correlation-Based Interpretation as Evidence of Causality

Throughout the paper, the authors interpret correlational differences as evidence of causality. This statistical misinterpretation permeates the metabolomics, transcriptomics and spatial analyses. For example, differences in metabolite abundance between zones are interpreted as mechanistic drivers of tumor susceptibility, despite the absence of flux-based evidence or perturbation experiments. Similarly, transcriptional differences identified in pseudoreplicated single-cell data are treated as independent predictors of malignant initiation.

These interpretational leaps violate basic statistical principles. Correlation does not imply causation, particularly in complex spatial systems where multiple biological and microenvironmental variables interact. The study lacks the experimental manipulations required to establish causal relationships, rendering its strongest claims statistically unsupported.

6.8. Summary of Statistical Limitations

The statistical weaknesses in Guo *et al*. undermine the central conclusions of the study. Insufficient biological replication, pseudo-replication in single-cell data, absence of multiple-testing correction, lack of spatial modeling, inadequate batch correction and failure to distinguish correlation from causation collectively render the statistical foundation of the paper unstable. These limitations propagate through every major result, from metabolic profiling to spatial lineage tracing, leaving the study's deterministic claims about HCC origin unsupported by rigorous statistical evidence.

7. Biological and Mechanistic Contradictions

7.1. Zonal Identity Is Not Fixed but Fluid, Undermining Any Deterministic Model of Tumor Origin

A fundamental mechanistic contradiction in the Guo *et al*. study[1] is the assumption that hepatocytes possess fixed zonal identities that persist long enough for malignant transformation to occur in a predictable, spatially restricted manner. Modern liver biology has demonstrated repeatedly that hepatocyte zonation is dynamic. Hepatocytes undergo transcriptional remodeling in response to oxygenation changes, nutrient fluctuations, circadian cycles, inflammatory signals and injury. This fluidity allows hepatocytes to shift their metabolic programs toward periportal-like or pericentral-like states as needed. Such plasticity is incompatible with deterministic origin models that require stable spatial identities.

The microenvironment further destabilizes zonation during the earliest stages of hepatocarcinogenesis. Oxidative stress, DNA damage accumulation and inflammatory signaling alter hepatocyte metabolism in ways that blur canonical zonal distinctions. Cells in zone 3 may adopt zone 1–like profiles under hypoxic stress, while zone 1 hepatocytes can acquire pericentral xenobiotic metabolism during toxic exposure. By ignoring the biologically established fluidity of zonation, the authors present a model inconsistent with known hepatocyte behavior.

7.2. Oncogenic Stress Rapidly Reconfigures Hepatocyte Metabolism, Erasing Zonal Signatures

The mechanistic framework proposed by Guo *et al*.[1] assumes that tumor-initiating hepatocytes retain their zonal metabolic signatures during the earliest steps of transformation. However, oncogenic stress induces profound metabolic reprogramming. Mutations in β-catenin, TERT promoter activation or TP53 loss lead to shifts in glycolysis, lipid metabolism, glutaminolysis and mitochondrial function that override baseline zonal differences. Early transformation stages involve activation of stress kinases, epigenetic remodeling and chromatin restructuring, all of which converge to produce transcriptional signatures distinct from any physiological zonal program.

Experimental evidence from lineage tracing of oncogene-induced clones shows that pretransforming hepatocytes lose their zonal identity as they undergo dedifferentiation and metabolic reconfiguration. During early carcinogenesis, Wnt pathway activation, oxidative stress and cytokine exposure erase zone-specific metabolic states. This erasure undermines any attempt to reconstruct origin based on zonal signatures. The assumption that zonal identity persists through

transformation is mechanistically unsubstantiated and contradicts the robust literature on metabolic reprogramming in early HCC development.

7.3. Hepatocyte Migration and Clonal Dynamics Contradict a Static Spatial Origin Model

Hepatocytes are not static entities anchored permanently to specific lobular territories. Recent studies employing genetic barcoding, mosaic tracing and spatial transcriptomics show that hepatocyte clones migrate along the porto-central axis over time. Clones derived from periportal regions can expand pericentrally during regeneration or chronic injury, and vice versa. Clonal replacement occurs in waves, driven by microenvironmental cues, tissue damage and metabolic demand.

These clonal dynamics create a major mechanistic conflict for the zone-of-origin hypothesis presented by Guo *et al*.[1] If a tumor emerges from a clone that has migrated between zones during the latency period preceding malignant transformation, then anatomical position at the time of detection does not reflect original spatial provenance. The authors attribute tumor origin to final location rather than the historical trajectory of the clone. A dynamic, migrating hepatocyte population cannot sustain the static mapping required by a zonation-dependent model. The study does not address this clonal behavior, rendering its spatial interpretations biologically incomplete.

7.4. Transformation-Induced Microenvironmental Changes Reshape Zonation and Confound Origin Mapping

The liver microenvironment is a potent regulator of zonation. Sinusoidal endothelial cells, stellate cells, Kupffer cells and infiltrating immune cells release signaling molecules that shape metabolic and transcriptional gradients. During early carcinogenesis, microenvironmental remodeling is profound. Cytokines such as IL-6, TNF-α and TGF-β alter hepatocyte metabolism. Stellate cell activation reshapes ECM composition and mechanical tension. Endothelial cells shift angiocrine output in response to oncogene activation.

These changes disrupt the baseline zonation patterns assumed by Guo *et al*. and may create new microdomains with altered metabolic profiles that do not correspond to classical zone 1, zone 2 or zone 3 territories. A hepatocyte that appears pericentral in location may actually exist within a microenvironment that has perverted zonation-like transcriptional states. The failure to assess microenvironmental zoning and stromal contributions makes it impossible to assign origin purely on hepatocyte markers, because these markers are themselves influenced by niche alterations induced by transformation.

7.5. Stem-Like Hepatocyte Subpopulations Are Scattered Across Zones, Not Confined to Specific Territories

A growing body of evidence indicates that hepatocytes with enhanced proliferative potential or stem-like features are distributed throughout the hepatic lobule rather than restricted to a specific zone. Cells expressing markers such as Tbx3, Sox9, Axin2 and other facultative progenitor signatures appear at multiple lobular locations, often enriched near the midzone or responding dynamically to injury. These cells may act as precursors to regenerative or neoplastic clones.

If stem-like hepatocytes are not zone-specific, then the zone-of-origin model proposed by Guo *et al*.[1] lacks biological grounding. A tumor arising from such a population cannot be attributed reliably to a specific metabolic zone, because the lineage competence associated with transformation is not spatially compartmentalized. The authors' failure to integrate these findings reflects a mechanistic oversimplification that undermines their deterministic framework.

7.6. Oxidative Stress, Mitochondrial Function and DNA Damage Responses Vary Temporally, Not Spatially

One of the mechanistic claims implied in Guo *et al*.[1] is that the metabolic states of different zones create intrinsic susceptibilities to DNA damage and transformation. However, oxidative stress and mitochondrial dysfunction fluctuate temporally in response to diet, circadian rhythms, intermittent hypoxia and toxin exposure. These fluctuations often exceed the baseline differences between zones.

Furthermore, DNA damage responses and repair efficiencies vary dynamically during cell cycle transitions and under inflammatory conditions. The temporal variability of these pathways means that at any given moment, susceptibility to transformation may be dictated more by transient microenvironmental cues than by spatial location. Guo *et al*. do not measure DNA damage markers or oxidative stress responses in premalignant hepatocytes across time. Without such measurements, their assumption that static zonal metabolic differences underlie transformation susceptibility remains mechanistically unsupported.

7.7. Incomplete Integration of Known Pathways in HCC Initiation

The study largely ignores the well-established oncogenic pathways implicated in HCC initiation, including Wnt signaling, Hippo pathway dysregulation, TERT promoter activation, MYC upregulation and JNK-mediated stress signaling. These pathways exert strong effects on hepatocyte proliferation and metabolic rewiring that overshadow zonal differences. By not integrating these pathways into the

model, the authors present an isolated view of HCC initiation that lacks coherence with broader cancer biology.

Additionally, many oncogenic drivers directly influence metabolic reprogramming, making it impossible to interpret metabolic differences as causes rather than consequences of transformation. The absence of perturbation experiments or mechanistic assays in Guo *et al*. leaves a conceptual vacuum that weakens the proposed model.

7.8. Summary of Mechanistic Contradictions

The deterministic zonation-dependent model proposed by Guo *et al*.[1] conflicts with well-established aspects of hepatocyte biology, including metabolic plasticity, oncogenic reprogramming, hepatocyte migration, microenvironmental modulation, clonal evolution and the distribution of stem-like progenitors. The mechanistic assumptions underlying the study are inconsistent with decades of research and are not substantiated by the data presented. Without reconciling these contradictions, the central claims of the study cannot be biologically credible.

8. Imaging, Data Integrity, and Reproducibility Concerns

8.1. Variability in Fluorescence Intensity and the Absence of Standardized Imaging Parameters

A pervasive issue throughout the imaging data presented by Guo *et al*.[1] is substantial variability in fluorescence intensity between panels, even within the same experimental condition. The study does not report critical imaging parameters such as exposure time, detector gain, laser power or photomultiplier tube settings. Without these details, it is difficult to determine whether observed differences in signal intensity reflect biological variation or technical inconsistencies. Some panels appear overexposed, leading to saturation of pericentral markers, while others appear underexposed, obscuring periportal staining. This inconsistency compromises the interpretability of zonal boundaries and creates uncertainty about whether the spatial gradients depicted in the figures are genuine or artefactual.

The absence of standardized imaging protocols also prevents reproducibility. Without access to raw confocal settings, subsequent investigators cannot replicate the imaging conditions. Moreover, uneven brightness across fields suggests that flatfield correction was either not applied or inconsistently used. These technical weaknesses elevate the risk of misinterpretation, especially in a study where subtle spatial gradients are central to the main conclusions. Given that zonation depends on continuous differences in marker expression rather than discrete boundaries, even small inconsistencies in image acquisition have major interpretational consequences.

8.2. Incomplete Description of Antibody Validation and Staining Procedures

Immunostaining forms the backbone of zonal identity assessment in this study, yet the methods section provides only minimal documentation regarding antibody validation, lot numbers, titration, or specificity assays. For markers such as glutamine synthetase, CYP2E1 and CPS1, lot-to-lot variability is common and can lead to different staining patterns. Without negative controls, peptide block assays or validation against known knockout tissues, it is unclear whether the observed staining genuinely reflects endogenous protein distribution.

Furthermore, the study does not describe whether the same antibodies were used across all experimental batches, nor whether the staining protocol was optimized for each marker's dynamic range. Immunostaining without proper validation introduces significant uncertainty, particularly in regions where marker expression is expected to be low or diffuse. The failure to report antibody validation limits confidence in the zonation patterns shown and makes independent verification impossible.

8.3. Possible Tissue Processing Artifacts and Their Impact on Zonation Interpretation

The liver is highly sensitive to fixation conditions, and variations in tissue processing can dramatically alter spatial staining patterns. The authors do not specify fixation times, fixative composition, permeabilization conditions or section thickness. Formaldehyde concentration and fixation duration influence antigen preservation differently across zones because periportal and pericentral regions have different protein turnover and lipid content. Over-fixation can reduce staining intensity in deeper tissues, leading to artificial flattening of zonation gradients.

Sectioning artifacts, including tearing, folding, compression and edge drying, are visible in several images. These artifacts distort sinusoidal architecture and may give the illusion of spatial boundaries that are not biologically present. For example, compressed tissue regions may mimic abrupt expression changes, while uneven section thickness can cause zonal markers to appear discontinuous. The absence of methodological clarity around tissue processing introduces significant doubt about the fidelity of the spatial patterns shown.

8.4. Lack of Confocal Z-Stacks, Depth Calibration and Three-Dimensional Imaging

A major limitation of the imaging data is the reliance on single optical planes rather than confocal z-stacks or three-dimensional reconstructions. Zonation is an inherently spatial phenomenon extending through the depth of the lobule. Single-

plane images cannot represent the complexity of local gradients, especially when tissue thickness exceeds the confocal depth of field. Without z-stack projections or volumetric reconstructions, it is impossible to determine whether marker expression gradients are consistent across depth or whether they represent artifacts of sampling plane orientation.

Depth calibration is also missing. Fluorescence intensity decreases with depth due to scattering, absorption and refractive index mismatch. Without correcting for these factors, deeper pericentral hepatocytes may appear dimmer than periportal cells, artificially reinforcing zonal patterns. In the absence of three-dimensional imaging, claims about spatial boundaries remain inferential rather than data-driven.

8.5. Potential Image Stitching Artefacts and Unexplained Repeated Features

Several panels, particularly in the supplementary figures, show abrupt transitions between regions that resemble stitching boundaries rather than biological interfaces. These transitions manifest as sudden changes in intensity, contrast or cellular morphology within a continuous field. In some panels, hepatocytes appear duplicated or possess nearly identical morphological features, suggesting that either automated stitching or manual cropping was performed without disclosure.

Image stitching is a legitimate technique for assembling large fields of view, but it requires careful reporting, including stitching algorithms, overlap parameters and correction for mismatched brightness at tile boundaries. The authors provide no such documentation. The presence of potential stitching artefacts undermines confidence in the spatial coherence of the images and opens the possibility that zonation boundaries may be exaggerated or misrepresented by technical artifacts rather than biological truth.

8.6. Absence of Raw Imaging Data for Independent Verification

Reproducibility demands access to raw imaging data, especially when claims rely heavily on the interpretation of subtle spatial features. The authors do not provide raw .czi, .lif, .nd2 or .tif stacks, nor do they include metadata such as voxel dimensions, z-step size or bit depth. Without raw files, independent investigators cannot inspect pixel-level features, intensity histograms or fluorescence distribution. This absence is particularly problematic given the concerns about uneven exposure, potential stitching artifacts and incomplete representation of depth.

The lack of raw data also prevents assessment of noise filtering, background subtraction and thresholding. These steps can dramatically alter the visibility of

gradient-based patterns. Without transparency, the possibility of inadvertent or intentional bias in image processing cannot be excluded.

8.7. Inconsistent Presentation of Scale Bars and Spatial Context

Across the main and supplementary figures, scale bars are inconsistently applied. Some panels include scale bars of unspecified length, while others omit them entirely. Without scale information, it is impossible to evaluate the size of hepatocytes, the width of lobular zones or the spatial relationship between central veins and periportal regions. In a study claiming spatial determinism, the absence of reliable scale calibration is a severe methodological flaw.

Moreover, several images lack labeling for key anatomical landmarks such as central veins, portal triads or midzonal boundaries. Without these landmarks, visual interpretation becomes highly subjective. Spatial context is essential for defining zones, and the lack of consistent orientation markers further complicates interpretation.

8.8. Concerns About Image Selection Bias and Incomplete Representation of Data

The figures presented appear to reflect a subset of the total images generated. There is no indication that the fields shown are representative of the full dataset. Given the variability observed across panels, the possibility of image selection bias cannot be dismissed. Images that conform to the expected zonation narrative may have been preferentially included, while inconsistent or contradictory images may not have been shown. The absence of full datasets, quantification across multiple fields and transparent reporting of field selection criteria raises concerns about whether the images accurately reflect the underlying biological variability.

Selection bias is particularly damaging when combined with unvalidated Cre drivers, mosaic labeling and inconsistent staining. A robust analysis would require quantification across all fields of view, but such quantification is absent. As a result, the presented images cannot reliably support claims of zonation-dependent lineage or tumor origin.

8.9. Summary of Imaging and Reproducibility Issues

The imaging data in Guo *et al*. suffer from uneven fluorescence intensity, incomplete antibody validation, potential tissue processing artifacts, lack of z-stacks, absence of raw data, inconsistent scale bars, stitching artefacts and possible selection bias. These weaknesses undermine the credibility of spatial interpretations and call into question the reproducibility of the entire imaging dataset. Given the central role of

spatial evidence in the claimed zonation-dependent model of HCC origin, the methodological opacity and technical inconsistencies in the imaging undermine the foundation on which the study builds.

9. Alternative Interpretations Supported by the Data

9.1. Metabolic States as Consequences, Not Causes, of Early Malignant Transformation

A central interpretive leap made by Guo *et al*.[1] is the conclusion that metabolic zonation determines hepatocellular carcinoma initiation. The visual, molecular and spatial data presented in the study can easily support an alternative explanation: that observed metabolic differences in purportedly "*zonal*" tumors reflect the metabolic reprogramming induced by early oncogenic events, not antecedent zonal predisposition. Malignant transformation universally triggers shifts toward glycolysis, lipogenesis, mitochondrial remodeling, glutamine dependence and oxidative stress responses. These changes often mimic the metabolic identities associated with zone 3 hepatocytes, regardless of true spatial origin. Thus, a tumor appearing pericentral-like in metabolic profile may simply reflect oncogene-driven reprogramming rather than a zone-3 origin.

The study's reliance on bulk metabolomics and incomplete single-cell transcriptomics further promotes this alternative interpretation. Both methods capture the metabolic consequences of transformation but cannot distinguish whether these consequences preceded or followed oncogenic initiation. Because Guo *et al*. fail to conduct temporal analyses or perturbative experiments that modulate metabolic states prior to transformation, it is more parsimonious to interpret the data as reflecting metabolic adaptation rather than predisposition.

9.2. Microenvironmental Cues as Primary Determinants of Susceptibility

The hepatic microenvironment is a complex system of gradients in oxygenation, nutrient delivery, immune surveillance, endothelial signaling and stromal interactions. These gradients often dominate hepatocyte behavior more strongly than intrinsic zonal identities. For example, the regions near central veins experience lower oxygen tension, higher concentrations of xenobiotic metabolites and greater oxidative stress, all of which can influence DNA damage and mutation acquisition. Meanwhile, periportal regions are exposed to immune-rich blood flow entering from the portal vein, which may influence the inflammatory milieu surrounding hepatocytes.

Given these factors, a plausible interpretation of the data is that microenvironmental conditions create variable susceptibility niches that shift

dynamically over time. Tumors appearing in periportal areas may have arisen there not because periportal hepatocytes possess intrinsic susceptibility but because the local microenvironment temporarily promoted survival or expansion of a mutated clone. Conversely, tumors near central veins may reflect transient hypoxic stress or nutrient conditions rather than fixed zonal predisposition. Because Guo *et al*.[1] do not measure microenvironmental parameters, their model cannot distinguish between intrinsic zonal susceptibility and extrinsic spatial influence. This gap leaves microenvironment-driven susceptibility as an equally viable, and in many ways more coherent, interpretation.

9.3. Hepatocyte Migration and Clonal Drift as Explanations for Apparent Spatial Patterns

Recent advances in lineage tracing demonstrate that hepatocytes migrate over time, particularly in response to injury, regeneration or metabolic perturbation. Clonal populations can drift along the porto-central axis during normal tissue turnover. Guo *et al*.[1] do not account for the possibility that transformed hepatocyte clones may move away from their original site of mutation during early expansion. As such, a tumor located in zone 1 may originate from a clone that initially arose in zone 2 or zone 3 but migrated before expansion. Conversely, a midzonal tumor may have arisen near a portal triad but shifted position within the lobule as proliferation distorted local tissue architecture.

The absence of temporal lineage tracing renders static anatomical mapping insufficient for determining origin. The most parsimonious interpretation of the data is that the spatial location of detected tumors reflects a combination of clonal migration, tissue remodeling and transformation-associated expansion rather than the site of initial mutation. Without serial reconstructions or dynamic lineage tracking, the authors' conclusions remain speculative, and clonal drift provides a robust alternative explanation that aligns with established hepatocyte biology.

9.4. Zonation Shifts During Liver Injury as Key Confounding Factors

Zonation is profoundly reshaped during liver injury, inflammation and regeneration. Zones 1, 2 and 3 undergo transcriptional and metabolic reprogramming depending on cytokine exposure, oxidative stress, toxin metabolism and metabolic load. During carcinogen exposure or genetic induction of transformation, hepatocytes undergo stress responses that blur classic zonal markers. Supplementary figures in the Guo *et al*. paper already display inconsistent zonation patterns, suggesting that zonal boundaries are not preserved during the experimental conditions used.

An alternative interpretation is that transformation-associated stress conditions produce zonation shifts that alter the transcriptional and metabolic profiles of early preneoplastic cells. Under this interpretation, tumors appearing "*zone-specific*" reflect zonation states induced by oncogenic stress rather than physiologically maintained zonation patterns. This dynamic reconfiguration provides a mechanistic explanation for why tumors across different zones can show similar metabolic signatures. It also aligns with the observation that metabolic states become hybridized during early transformation. The authors' failure to evaluate zonation under stress conditions leaves this alternative interpretation more strongly grounded than the deterministic model they propose.

9.5. Injury-Induced Progenitor-Like Cells as Potential Tumor Initiating Populations

Another plausible interpretation of the data is that a population of facultative progenitor-like hepatocytes, which emerge in response to injury, serves as the origin of HCC. These cells appear in multiple zones, often enriched in areas undergoing stress or regeneration. Their distribution does not map cleanly onto classic zonation architecture. They exhibit high proliferative potential, activation of stress-responsive transcriptional programs and partial loss of zonal identity. Many lineage-tracing studies have demonstrated that these progenitor-like states can arise from any zone under appropriate conditions.

Given this, HCC may originate from hepatocytes transitioning through progenitor-like states rather than from specific zonal identities. The metabolic signatures captured by Guo *et al*.[1] could reflect the progenitor-like state rather than zone-specific identity. The spatial clustering of tumors near certain zones may simply reflect where progenitor activation occurred during the specific injury or transformation model used. This explanation is compatible with observations in human HCC, where tumors often emerge from regions enriched in regenerative nodules or ductular reactions rather than from metabolically defined zones.

9.6. Tumor Position Driven by Vascular and Hemodynamic Factors Rather Than Zonal Identity

Another alternative explanation arises from the liver's vascular architecture. Tumors frequently arise in regions with altered hemodynamics, reduced perfusion, or increased metabolic stress. Zone 3 hepatocytes experience lower oxygen tension and increased exposure to xenobiotics, while periportal hepatocytes encounter portal venous blood enriched in nutrients and immunomodulatory molecules. Spatial differences in perfusion, shear stress and sinusoidal flow patterns may influence mutation rates, inflammatory exposure and microenvironmental

pressures. These factors can shape tumor emergence independently of intrinsic zonal identity.

Guo *et al*.[1] do not assess vascular determinants. They do not measure oxygen gradients, perfusion rates or vascular remodeling during transformation. Without these measurements, attributing spatial tumor patterns to zonation rather than hemodynamic factors is unjustified. Hemodynamic susceptibility provides a plausible, physiologically grounded alternative explanation that the study does not consider.

9.7. Hybrid Metabolic States in Early Transformation as a Source of Confusion

Early oncogenic transformation frequently produces hybrid metabolic states that combine features of multiple zones. For instance, transformed hepatocytes may upregulate glycolysis while simultaneously enhancing oxidative phosphorylation, a pattern not characteristic of any specific zone. These hybrid states arise from oncogenic pressure rather than zonal identity. The presence of such hybrid states would confound any attempt to use metabolic signatures to infer origin. The single-cell RNA-seq data in the study show overlapping transcriptional profiles among early transformed cells, supporting the possibility that hybrid metabolic states exist and undermine zonation-based interpretation.

Under this alternative interpretation, the metabolic differences observed by Guo *et al*. are not indicators of origin but reflections of heterogeneous metabolic adaptation. The authors' conceptual framework does not accommodate hybrid states, but these states offer a more plausible explanation for the observed molecular patterns.

9.8. Summary of Alternative Interpretations

The data presented by Guo *et al*.[1] support multiple interpretations that do not rely on deterministic zonation-dependent origins of hepatocellular carcinoma. Metabolic differences may arise from transformation, not predisposition. Microenvironmental factors may govern susceptibility. Clonal migration and tissue remodeling may distort apparent spatial origins. Zonation shifts during injury confound interpretation. Progenitor-like states and hemodynamic factors may play dominant roles. Hybrid metabolic states complicate spatial inference. These possibilities align more closely with established liver biology than the deterministic model offered by the authors. In light of these alternative interpretations, the zonation-based origin model appears at best incomplete and at worst fundamentally flawed.

10. Synthesis and Integrative Assessment

10.1. Integration of Conceptual, Methodological, and Statistical Weaknesses

A coherent evaluation of Guo *et al*.[1] requires synthesizing the conceptual, methodological, statistical and imaging-related critiques into a unified assessment. When integrated, these analyses reveal that the study's foundational claim—that hepatocellular carcinoma originates in a zonation-dependent manner—is unsupported by the totality of the evidence. The conceptual model is based on assumptions that conflict with fundamental principles of hepatocyte biology, including metabolic plasticity, microenvironmental modulation and the dynamic nature of hepatocyte identity. Methodologically, the study employs tools that cannot resolve the spatial precision required to attribute tumor origin to specific metabolic zones. Zonal Cre drivers are not validated for fidelity, bulk metabolomics is interpreted as cell-intrinsic, and spatial mapping relies on two-dimensional sections of three-dimensional structures. Statistically, the reliance on pseudo-replicated single-cell data, underpowered metabolomic analyses, and uncorrected multiple comparisons produces spurious patterns that are then overinterpreted as biologically meaningful. Imaging inconsistencies further erode confidence in the spatial claims.

When viewed in isolation, each category of weakness might suggest a need for refinement. When considered collectively, they form a pattern of insufficient rigor that invalidates the deterministic conclusions. The integrative assessment thus requires recognizing that the study's shortcomings are not peripheral technical issues but fundamental gaps that undermine all higher-level inferences.

10.2. Incompatibility Between the Data and the Asserted Deterministic Model

The deterministic model proposed by the authors assumes that zone-specific hepatocytes possess intrinsic, stable susceptibility to malignant transformation. However, the data do not demonstrate such intrinsic predisposition. Instead, the patterns observed across figures can be explained by more parsimonious mechanisms, including transient metabolic reprogramming, microenvironment-induced susceptibility or stochastic clonal expansion. The lineage-tracing experiments do not establish a strict correspondence between Cre-labeled hepatocytes and subsequent tumors. The metabolomics results do not demonstrate clear zonal clusters. The single-cell transcriptomic maps do not show lineages maintaining stable zone-specific profiles during early oncogenic events.

The deterministic model also requires that tumors arising from specific zones retain metabolic signatures that reflect their origin. Yet early transformation induces metabolic rewiring that erases baseline zonal characteristics. The authors' interpretation of metabolic signatures as evidence of zonal origin disregards this known biological phenomenon. The inability of the dataset to distinguish between cause and consequence makes the assertion of deterministic origin unsustainable. The data are more consistent with a non-deterministic or context-dependent model of HCC initiation, where location influences susceptibility in a dynamic and reversible manner.

10.3. Internal Inconsistencies that Disrupt Narrative Coherence

Several internal inconsistencies emerge when comparing textual descriptions with visual and quantitative data. Zonal marker staining in supplementary figures does not match the clean, sharply delineated zonal boundaries described in the text. Lineage-tracing results show mosaic, diffuse patterns that conflict with the claim of zone-restricted recombination. Tumor mapping in main figures spans multiple zones despite being labeled as zone-specific in the narrative. Single-cell clusters overlap substantially even though they are described as transcriptionally distinct zonal lineages. Such inconsistencies demonstrate a disconnect between the data generated and the conclusions drawn.

Interpretational inconsistencies further erode confidence. Certain tumors are labeled as periportal in one panel yet appear centrally located in another without explanation. Regions of ambiguous marker expression are interpreted as definitive evidence of zonal identity while regions inconsistent with the proposed model are not discussed. These patterns suggest selective interpretation rather than objective assessment of the entire dataset. Narrative coherence collapses when key constructs, such as zonation stability and lineage fidelity, are contradicted by the figures intended to support them.

10.4. Missing Experiments That are Essential for Establishing Causality

A robust assessment of causality requires demonstrating that altering zonal metabolic states directly affects transformation susceptibility. The study does not include perturbation experiments that reprogram zonal identity or modulate zonal metabolic pathways during tumor initiation. Without targeted manipulations, such as inducing Wnt activation in periportal hepatocytes or modulating β-oxidation gradients, the claim of causal zonation cannot be validated.

Equally problematic is the absence of temporal mapping of zonation during oncogenesis. Without longitudinal imaging or molecular profiling, it is impossible to establish whether zonation precedes transformation or is reshaped by it. The lack of data on microenvironmental influences also leaves a mechanistic void. Given that the liver's immune, stromal and endothelial architecture undergoes dramatic reorganization during early transformation, ignoring these factors precludes causal interpretation. Perturbation-based validation, temporal mapping and microenvironmental modeling are essential to demonstrate causality. Their absence is not a minor flaw but an omission that leaves the central hypothesis unsupported.

10.5. The Broader Implications for Spatial Biology and HCC Research

The shortcomings of Guo *et al*.[1] have ramifications beyond the specific claim of zonation-dependent tumor origin. Spatial biology research increasingly relies on sophisticated techniques such as spatial transcriptomics, deep-learning image analysis and high-content lineage tracing. These technologies demand rigorous validation, transparent data reporting and careful interpretation to avoid conflating spatial correlation with causation. The missteps in this study highlight the importance of distinguishing spatial location from lineage identity, metabolic phenotype from molecular predisposition, and anatomical position from evolutionary history.

In HCC research, oversimplifying tumor origin risks obscuring the complex interplay between genomic alterations, metabolic reprogramming and microenvironmental forces. Deterministic spatial models may mislead therapeutic development if they suggest that specific hepatocyte subpopulations are fixed targets for intervention. Precision targeting of metabolic states requires understanding their plasticity and context dependence. The failure of Guo *et al*. to account for these factors underscores the need for more integrative approaches.

Moreover, the study's limitations point to the importance of multi-modal validation in spatial cancer biology. Reliance on any single modality—whether lineage tracing, metabolomics or single-cell profiling—invites misinterpretation in complex systems such as the liver. Future research must integrate multimodal datasets with careful statistical modeling and temporal resolution to elucidate the origins of HCC with the nuance and accuracy the problem requires.

10.6. A Coherent Synthesis: Why the Central Claim Cannot Be Sustained

Synthesizing the conceptual, methodological, statistical and imaging critiques leads to a clear conclusion: the evidence presented by Guo *et al*. does not support the

deterministic zonation-dependent model of hepatocellular carcinoma origin. The study lacks the spatial resolution, lineage fidelity, metabolic specificity and statistical rigor required to substantiate such a claim. The data do not demonstrate stable zonal identity during transformation, nor do they provide causal evidence linking zonation to malignant initiation. Alternative interpretations consistent with established hepatocyte biology are more plausible and better explained by the observations present in the dataset.

Thus, the central claim collapses under integrative scrutiny. While the study raises interesting questions about the relationship between liver architecture and cancer susceptibility, its conclusions represent an overreach of the available evidence. The synthesis presented here reaffirms the need for rigorous spatial methodology, transparent statistical analysis and biologically grounded interpretation in future investigations of tumor origin in the liver.

11. Conclusion

11.1. Summary of the Central Findings of This Commentary

The analysis presented in this commentary reveals that the central claim of Guo *et al*.[1]—that HCC originates in a zonation-dependent manner driven by intrinsic metabolic states of specific hepatocyte populations—is not supported by the evidence in the published article. Across conceptual framing, methodological execution, statistical design, imaging rigor and interpretational logic, the study falls short of the evidentiary standards required to assert deterministic spatial origins for a complex malignancy like HCC. The failures identified are not minor technical details but foundational structural weaknesses that undermine the central thesis. They include the assumption of static zonal identity despite extensive literature documenting metabolic plasticity, the unvalidated use of zonal Cre drivers with clear evidence of leakage, the use of two-dimensional histology to address inherently three-dimensional spatial questions and the reliance on pseudo-replication and underpowered datasets to generate molecular conclusions. The cumulative effect of these deficiencies is a narrative that exceeds the limits of the underlying data.

11.2. Why the Deterministic Zonation Model Cannot Be Sustained

The deterministic model proposed by Guo *et al*.[1] requires that hepatocytes maintain stable zonal identities during the earliest phases of malignant transformation and that these identities dictate susceptibility to oncogenic events. However, the integrated literature on liver biology demonstrates that hepatocytes constantly remodel their metabolic states in response to injury, inflammation, regenerative

demand and environmental conditions. Zonation is dynamic at baseline and is dismantled quickly during early carcinogenic stress. The lineage-tracing data provided in the study show mosaic and inconsistent labeling patterns incompatible with strict zonal restriction. The bulk metabolomic data, interpreted as evidence of intrinsic zonal susceptibility, instead reflect metabolic readouts confounded by multiple cell types and lack causal resolution. The single-cell RNA-seq data fail to show stable zonal transcriptional identities, while tumor mapping does not employ three-dimensional reconstruction, rendering origin assignments speculative. These fundamental issues collectively invalidate the deterministic zonation-dependent model.

11.3. The Broader Lessons for Spatial and Cancer Biology

The limitations of the study highlight broader methodological and conceptual issues facing the field of spatial cancer biology. As technologies such as spatial transcriptomics, lineage tracing, high-resolution imaging and single-cell profiling become more sophisticated, there is a growing temptation to interpret spatial patterns as causal determinants of tumor origin or evolution. However, spatial correlation must not be conflated with lineage identity or mechanistic susceptibility. The liver is an organ where positional information, metabolic state and microenvironmental cues interact in complex, dynamic ways. Studies seeking to draw deterministic conclusions must employ rigorous spatial modeling, comprehensive lineage-tracing validation and causal perturbation experiments to support such claims. Guo *et al*.[1] do not meet these standards, and their study illustrates the risks of interpreting spatial gradients without accounting for temporal dynamics, microenvironmental heterogeneity and biological plasticity.

The study also underscores the importance of methodological transparency and reproducibility. Imaging studies require detailed reporting of acquisition parameters, antibody validation, tissue processing and raw data availability. Lineage-tracing studies must quantify recombination fidelity and validate spatial specificity. Omics analyses must address pseudoreplication, batch effects and multiple-testing corrections. Failure to address these essentials leads to narratives that cannot stand up to scrutiny, even when the conceptual framework appears attractive.

11.4. Pathways for Future Research That Could Resolve Outstanding Questions

While the study's conclusions are unsupported, the overarching question it raises—how spatial architecture shapes hepatocyte susceptibility to malignant transformation—is scientifically important. Future research addressing this question must integrate temporal dynamics, microenvironmental signals and multi-

modal lineage tracing. High-resolution three-dimensional reconstructions using cleared liver tissue, light-sheet microscopy or multiplexed spatial transcriptomics will be essential for accurately mapping clonal origins. Single-cell analyses must be performed with appropriate biological replication and pseudobulk statistical frameworks. Zonal identity must be assessed longitudinally during early oncogenic events using reporters that remain active during stress and transformation. Metabolic susceptibility must be interrogated using isotope-based metabolic flux analysis rather than abundance-only profiling. Causality must be tested using targeted perturbations that shift zonation boundaries or metabolic programs to see whether these manipulations alter susceptibility to transformation.

These approaches will provide a stronger foundation for understanding whether certain regions of the hepatic lobule are indeed predisposed to oncogenesis or whether susceptibility is governed by transient environmental conditions, clonal dynamics or stochastic events. Incorporating environmental, genetic and metabolic heterogeneity into these models will also be essential.

11.5. Final Evaluation

In its current form, the evidence presented in Guo *et al*.[1] does not substantiate the claim that HCC origin is determined by metabolic zonation. The study's conceptual assumptions are inconsistent with established hepatocyte biology, the methodological execution lacks rigor, the statistical analyses are fundamentally flawed and the imaging data do not support precise spatial interpretation. The deterministic conclusion advanced by the authors cannot withstand detailed scrutiny. Although the study raises interesting questions and contributes to ongoing discussions about the role of spatial biology in cancer initiation, the claims it makes are substantially overstated relative to the quality of evidence provided.

The field will benefit from future studies that apply more rigorous lineage tracing, quantitative spatial mapping, mechanistic perturbation and multi-dimensional integration. Only through such approaches can we hope to elucidate the complex interplay between hepatocyte identity, microenvironmental forces and malignant transformation.

Reference

1 Guo, J. *et al.* The origin of hepatocellular carcinoma depends on metabolic zonation. *Science*, eadv7129 (2025). https://doi.org/10.1126/science.adv7129

2 Yasaka, T. M., Kim, C. K., Meadows, V. & Monga, S. P. Zonation, zonation, zonation: The real estate of the liver. *Annu Rev Pathol* (2025). https://doi.org/10.1146/annurev-pathmechdis-042624-091820

3 Halpern, K. B. *et al.* Single-cell spatial reconstruction reveals global division of labour in the mammalian liver. *Nature* **542**, 352-356 (2017). https://doi.org/10.1038/nature21065

4 Ben-Moshe, S. *et al.* Spatial sorting enables comprehensive characterization of liver zonation. *Nat Metab* **1**, 899-911 (2019). https://doi.org/10.1038/s42255-019-0109-9

5 Galasso, L. *et al.* Hepatocellular carcinoma and the multifaceted relationship with its microenvironment: attacking the hepatocellular carcinoma defensive fortress. *Cancers (Basel)* **16** (2024). https://doi.org/10.3390/cancers16101837

6 Cuesta, A. M. *et al.* New and old key players in liver cancer. *Int J Mol Sci* **24** (2023). https://doi.org/10.3390/ijms242417152