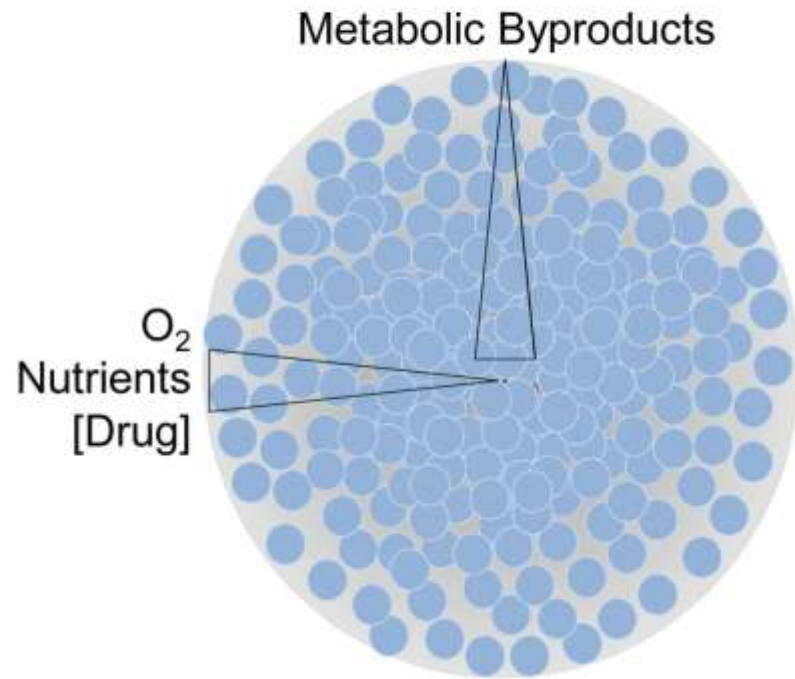


## INTRODUCTION

While drug response profiling of cancer cells in two-dimensional culture has been a mainstay of predictive biomarker discovery and anti-cancer drug development, there are aspects of tumor biology that are not replicated in a two-dimensional cell culture environment. This is particularly true for solid tumor models where the three-dimensional organization of the tumor creates distinct, regional microenvironments that influence drug response. For example, as the radius of solid tumors approaches the diffusion limit of oxygen and other nutrients, the tumor core becomes hypoxic and accumulates metabolic waste products, resulting in cell death through mechanisms of apoptosis and necrosis. Moreover, without proper vascularization, the tumor interior also exhibits increased interstitial pressure, which limits drug penetration into the tumor. We have characterized the three-dimensional growth of 240 cancer cell lines from various tumor origins by high-content imaging. This characterization involves the growth of tumor spheroids over 14 days and an assessment of tumor hypoxia in each model. The genomic traits associated with spheroid formation are also defined through genomic analysis. Finally, we provide validation and drug response data for reference cancer therapeutics in pre-formed, three-dimensional tumor spheroid models. Drug profiling of this three-dimensional cell line panel offers new insights into drug response and can be used to prioritize drugs with improved tumor penetration or those with otherwise improved activity in three-dimensional models.

**Figure 1. Cell line performance under long-term culture conditions. (A) Schematic illustrating the concentration gradients experienced in three-dimensional (3D) tumor spheroids. (B) 384-well Cell-Able Oncology plates were used to culture tumor spheroids. 250 collagen-coated spots are arrayed within each well, which serve as points of attachment for the nascent spheroids. Each spot is 100  $\mu\text{m}$  in diameter, similar to the diffusion limit of oxygen.**

**(A)**

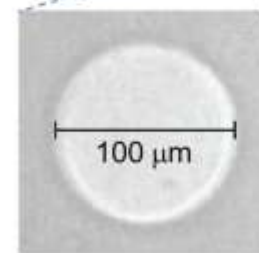
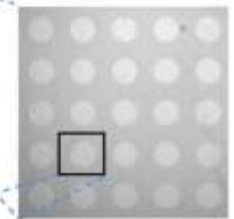


**(B)**

384-well  
Cell-able Oncology plate

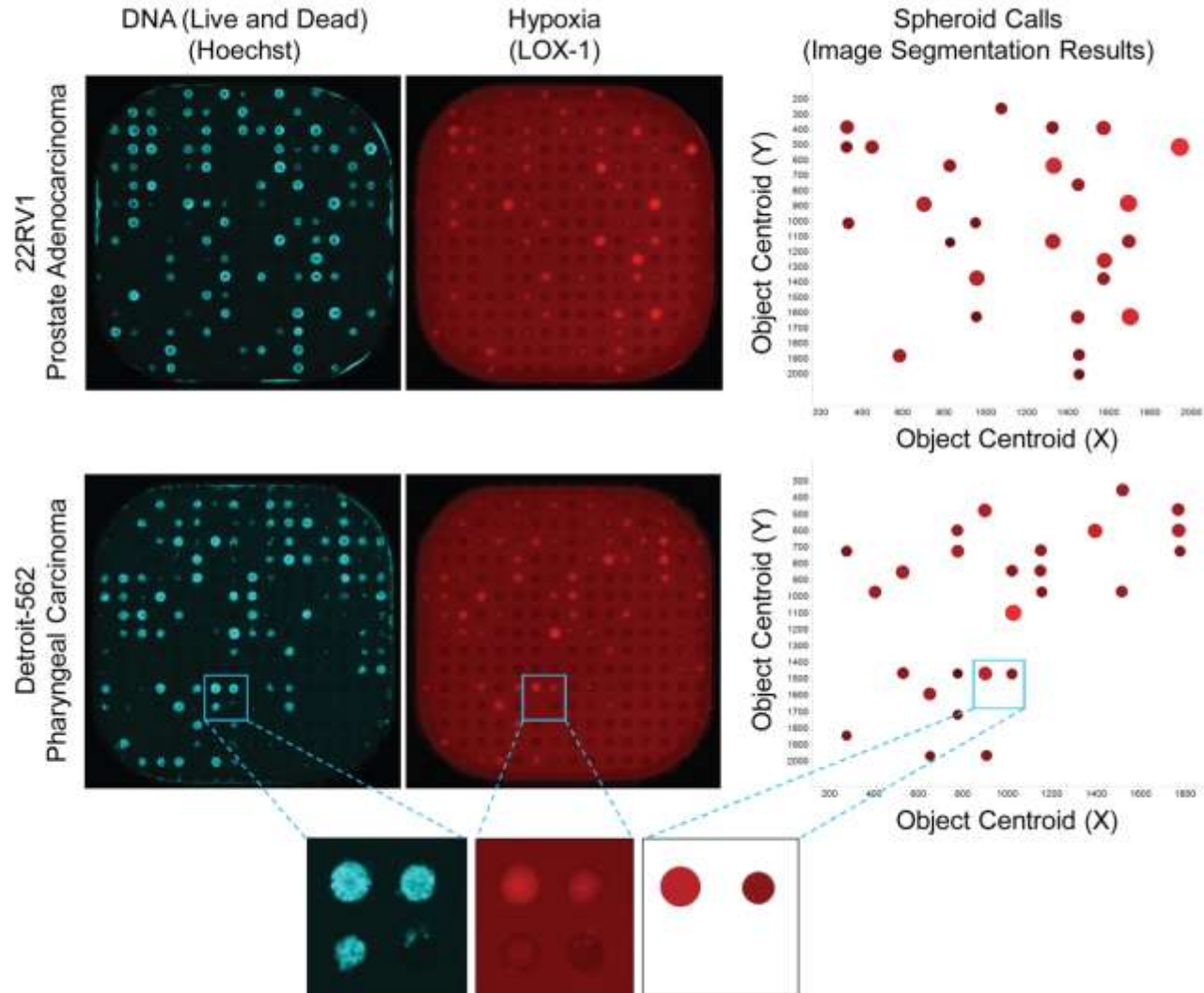


Array of 250 collagen-coated spots per well



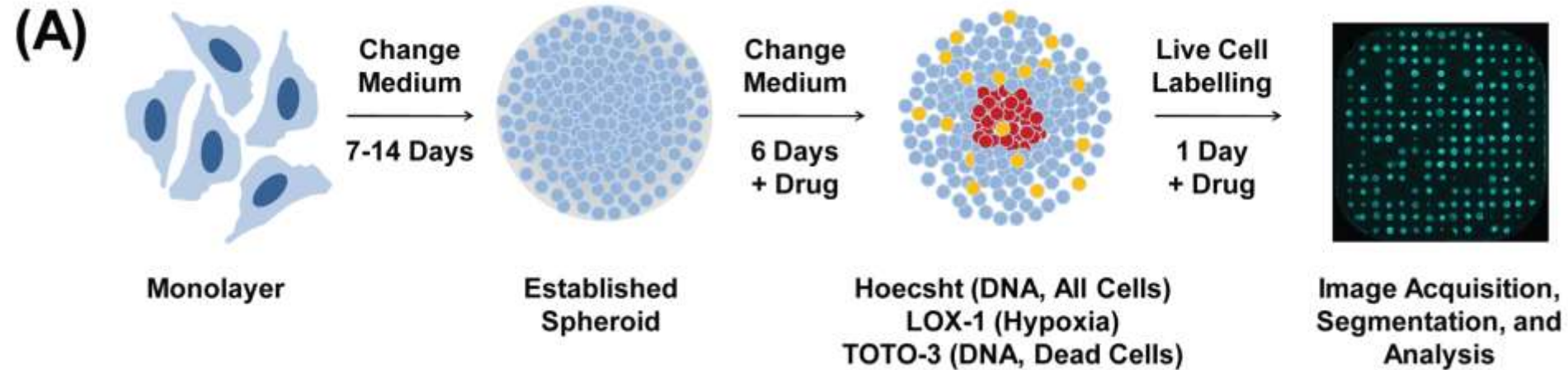
**Figure 1. Cell line performance under long-term culture conditions. (C) Whole well images of tumor spheroids after 14 days in culture and stained with Hoechst, a cell-permeable DNA stain and LOX-1, which produces phosphorescence in the absence of oxygen. The position of each spheroid in the well is defined by the Cartesian coordinate of its centroid. Spheroid calls after image segmentation are plotted on the right. Size and color of each spheroid is by Hoechst integrated intensity.**

**(C)**



**Figure 2. Methodology and Characterization of 3D cultures in 240 Cell Lines.**

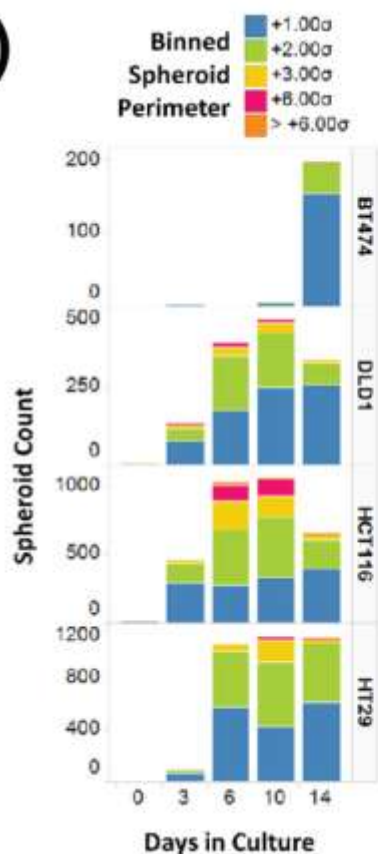
**(A) The OncoPanel™ collection of 240 cancer cell lines was cultured over a 14 day period after seeding cells at 200/well (just under 1 cell per spot) to establish spheroids. Drug treatments, where applicable, were initiated after a 7- or 14-day period, and incubations were performed for an additional 7 days. During the last 24 hr, live spheroids were labeled with the indicated stains at non-toxic concentrations and incubated for an additional 24 hr prior to image acquisition and analysis.**



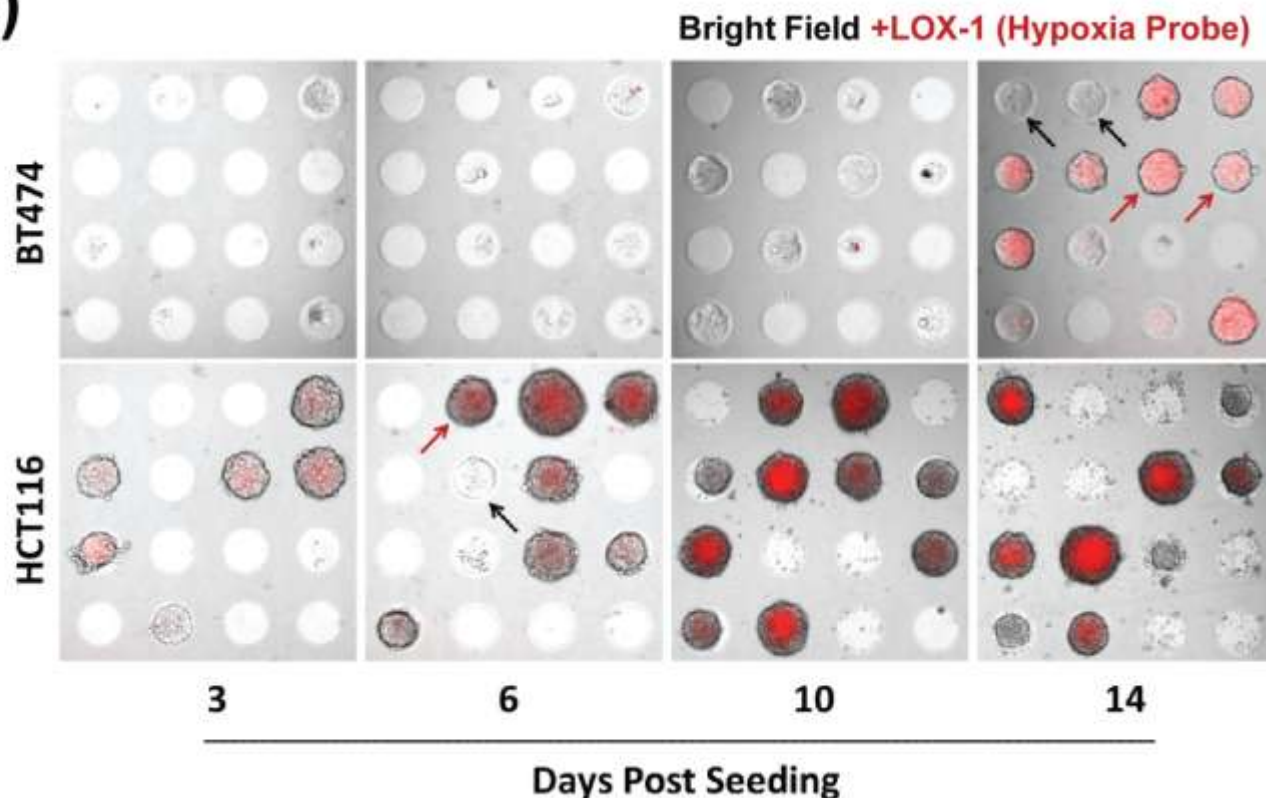
**Figure 2. Methodology and Characterization of 3D cultures in 240 Cell Lines.**

**(B and C) Spheroid size over time for cell lines with different 2D growth rates. Spheroids are defined by morphology and LOX-1 positivity.**

**(B)**



**(C)**





**Figure 2. Methodology and Characterization of 3D cultures in 240 Cell Lines.**  
**(D and E) Classification of 3D growth characteristics by tissue of origin.**

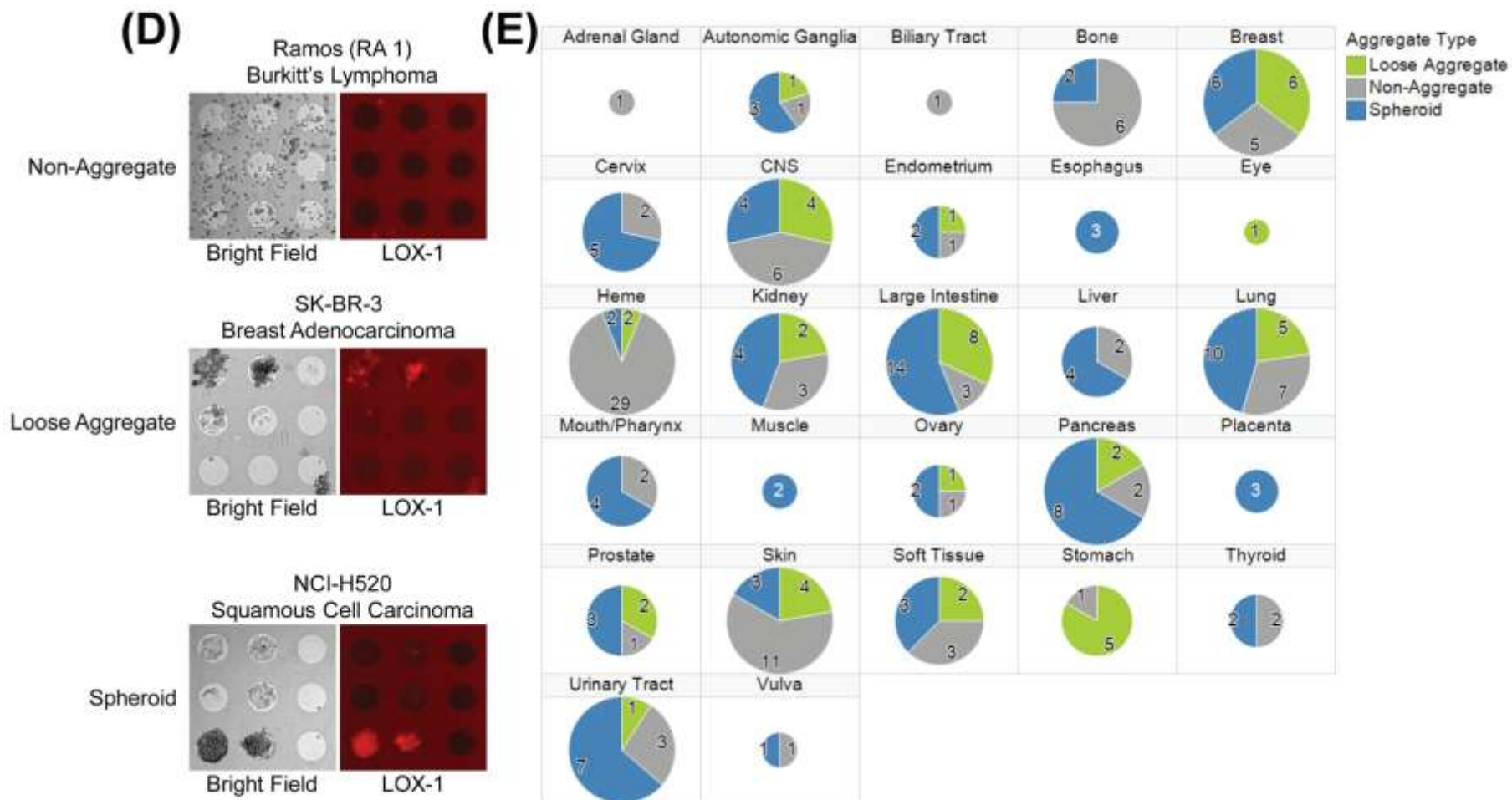
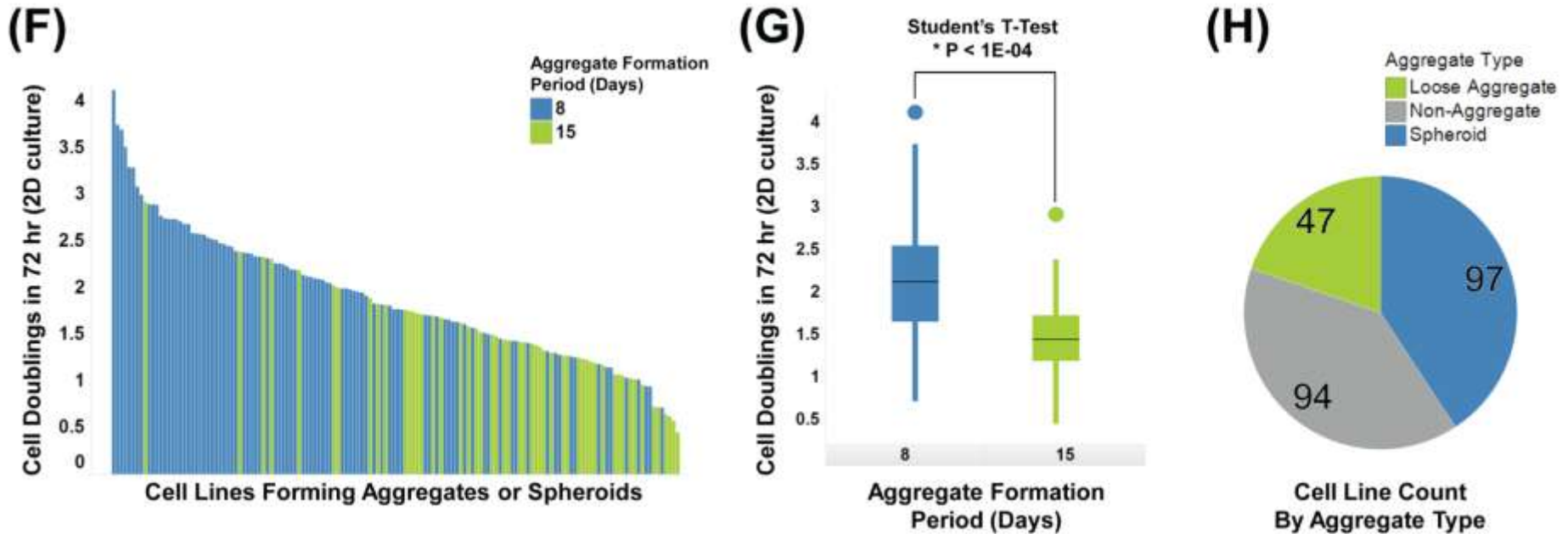
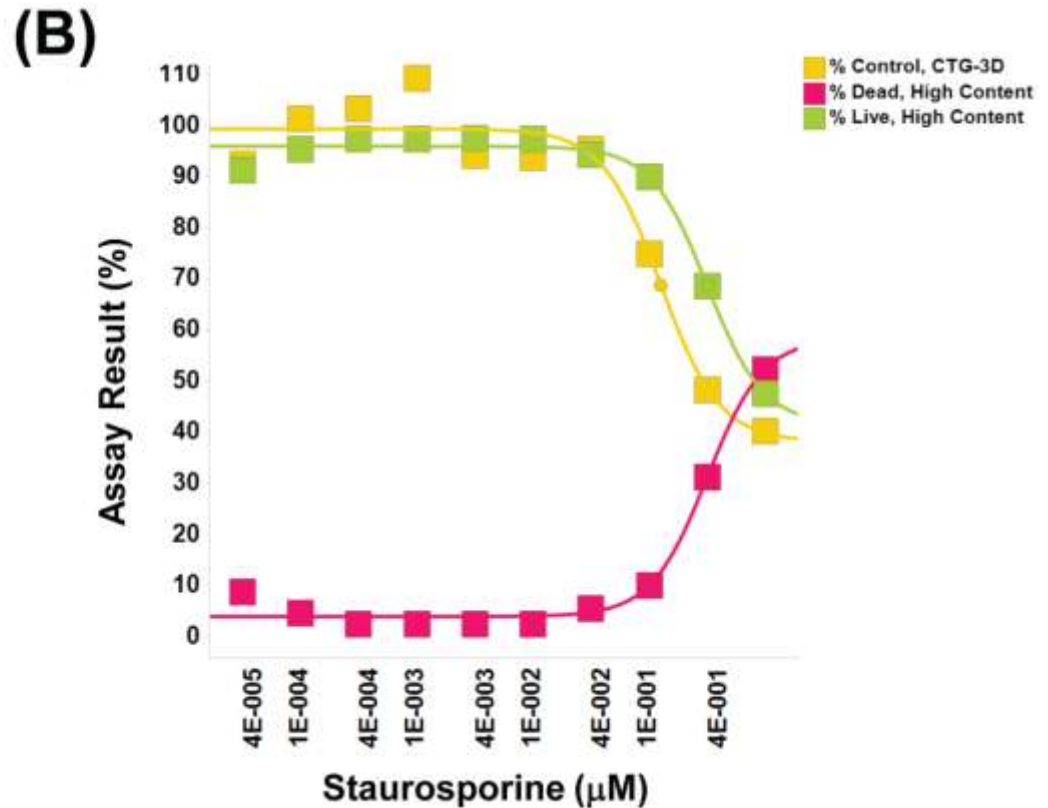
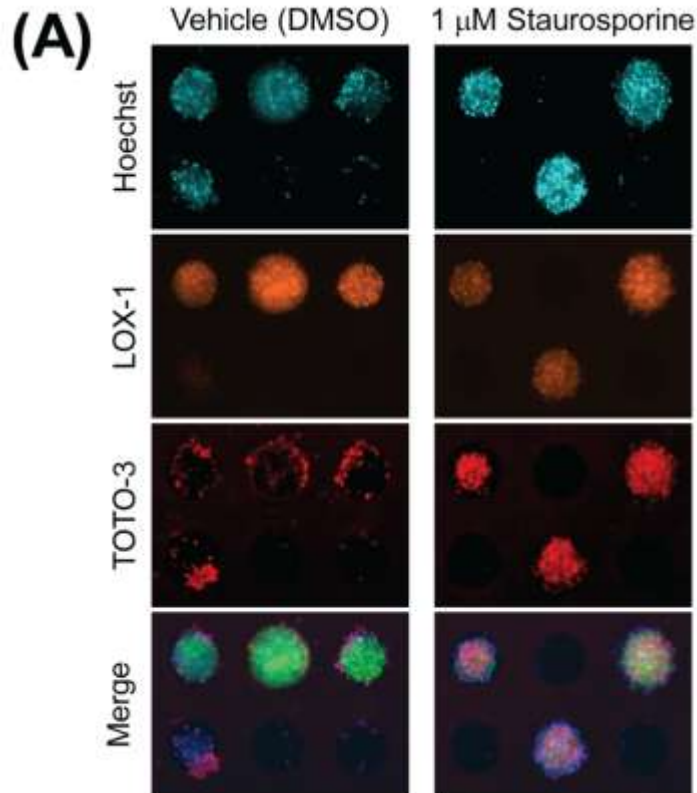


Figure 2. Methodology and Characterization of 3D cultures in 240 Cell Lines.

(F and G) Relationship between 2D doubling rate and time required for spheroid formation. Slower growing cell lines require more time in culture to form spheroids. (H) Number of OncoPanel™ cell lines by 3D growth characteristics.



**Figure 3. Inhibition of spheroid growth as measured by high-content imaging and metabolic activity. (A) High-content imaging of NCI-H460 spheroids after 7 days exposure to vehicle or 1  $\mu$ M staurosporine. Hoechst is a cell-permeable DNA stain (live and dead cells), LOX-1 produces phosphorescence in the absence of oxygen, and TOTO-3 is a cell-impermeable DNA stain (dead cells). (B) Staurosporine dose response curves for fraction live and dead by high-content imaging compared to CellTiterGLO-3D, which reports metabolic activity of viable cells.**





**Figure 3. Inhibition of spheroid growth as measured by high-content imaging and metabolic activity. (C) Representative bright field and LOX-1 fluorescent images of WiDr and NCI-H460 demonstrating that the majority of cells exist in spheroids. (D) Dose response curves for four inhibitors with distinct mechanisms of action generated after 7 days of treatment either in 2D or 3D cell culture. Note, the difference between these two culture conditions is more pronounced, in general, for NCI-H460 than for WiDr with some, but not all, inhibitors.**

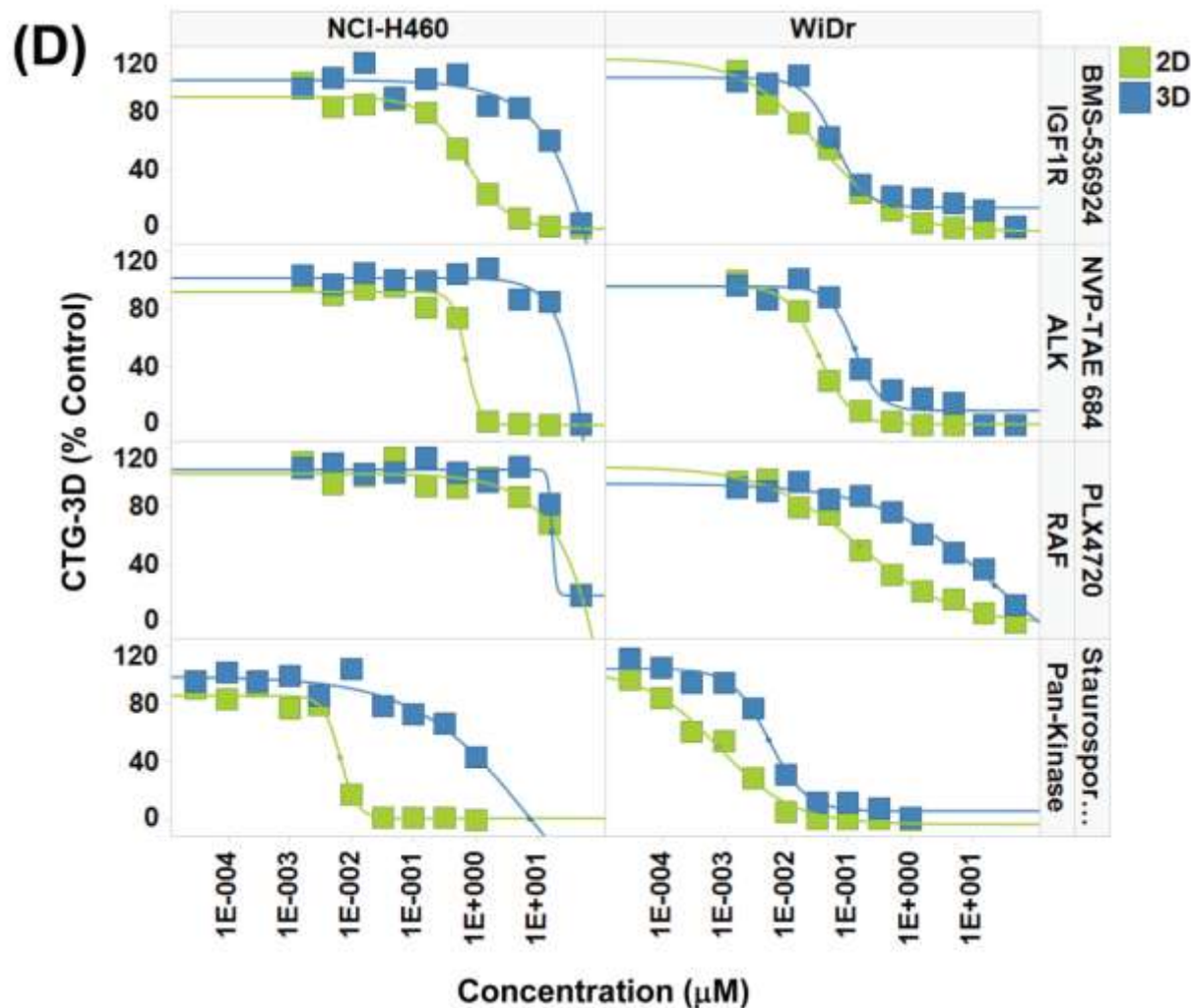
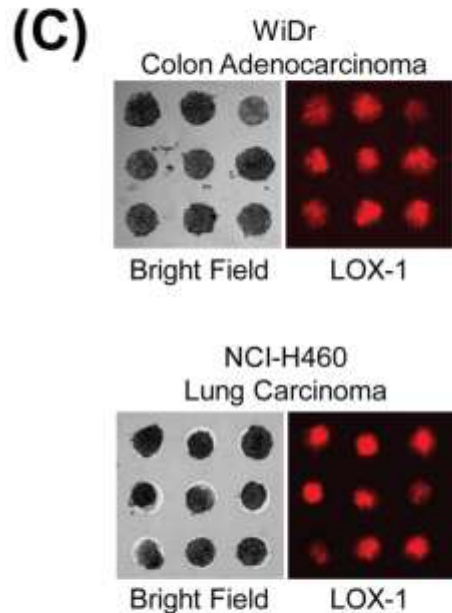


Figure 4. Genomic traits necessary for spheroid formation. (A) Spheroid formation was assessed in 240 cancer cell lines based on morphology and LOX-1 positivity after 14 days in culture. 94 cell lines did not grow as aggregates and were excluded from further analysis. 144 cell lines were classified as either loose aggregates or spheroids. An analysis was performed to identify genomic traits associated with the ability to form spheroids in culture

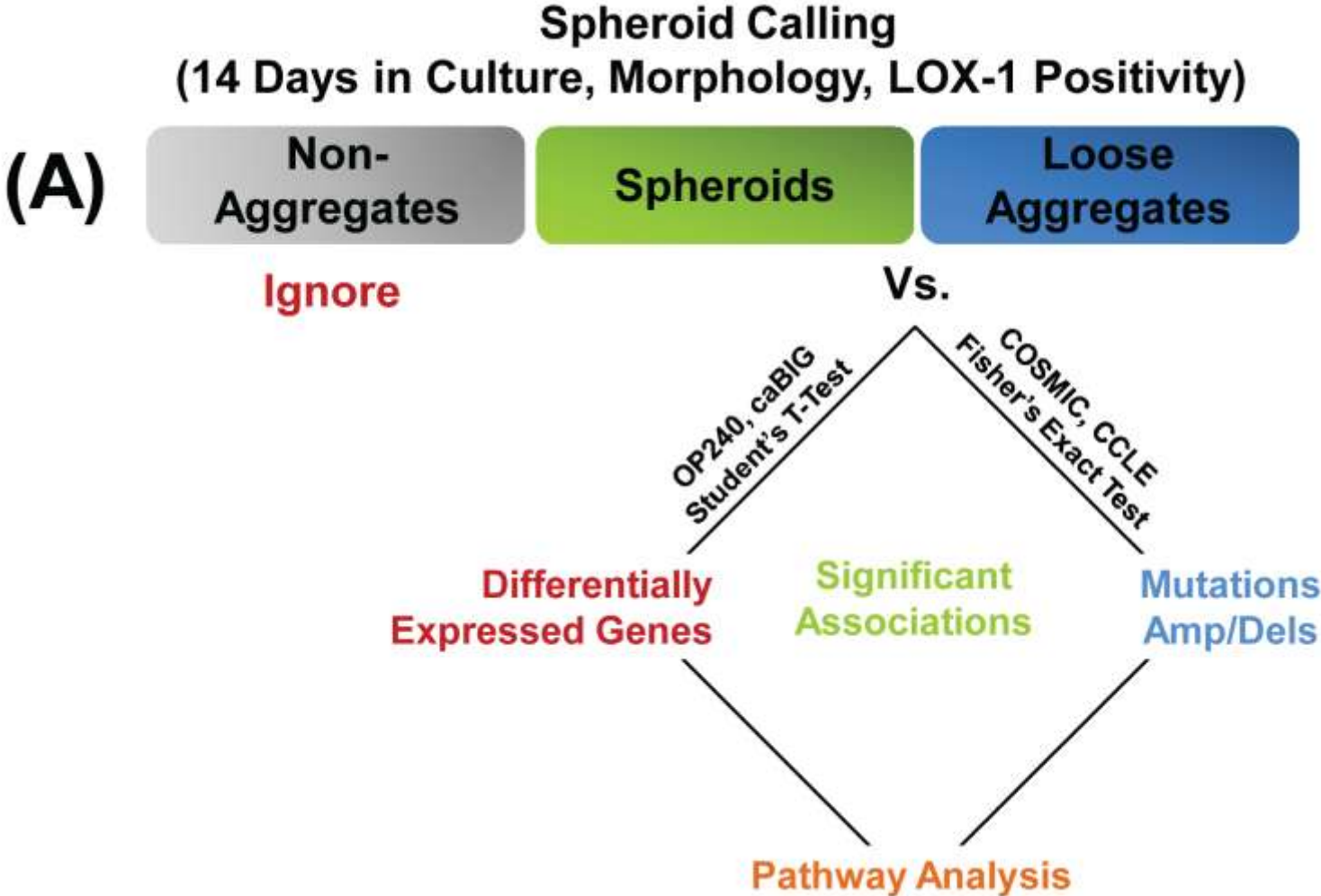
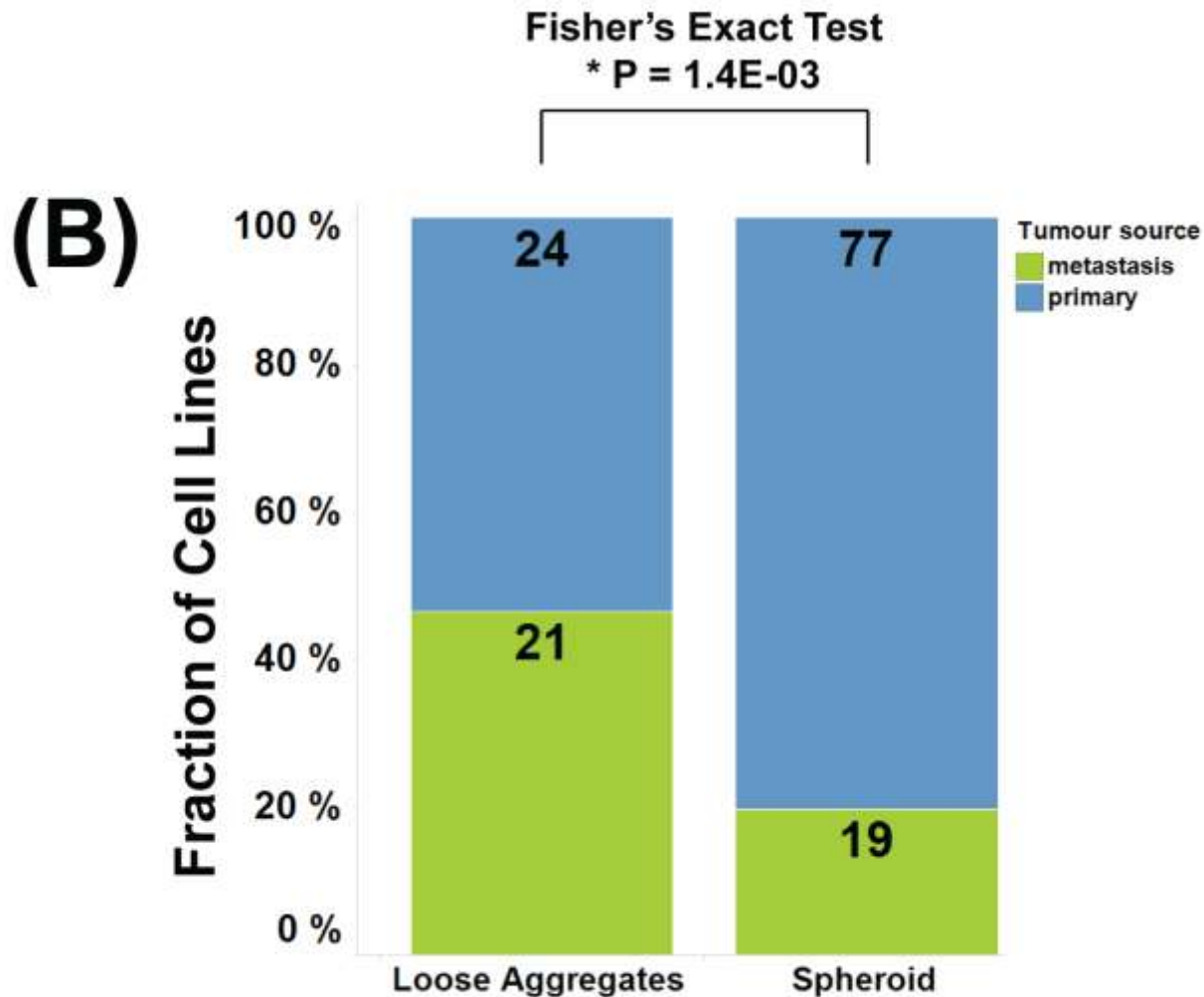


Figure 4. Genomic traits necessary for spheroid formation (B) Cell metastatic tumors tend to grow as loose aggregates in 3D culture.



**Figure 4. Genomic traits necessary for spheroid formation. (C) Differential mRNA expression analysis reveals an enrichment in genes associated with cell-cell junction formation and actin dynamics.**

**(C)**

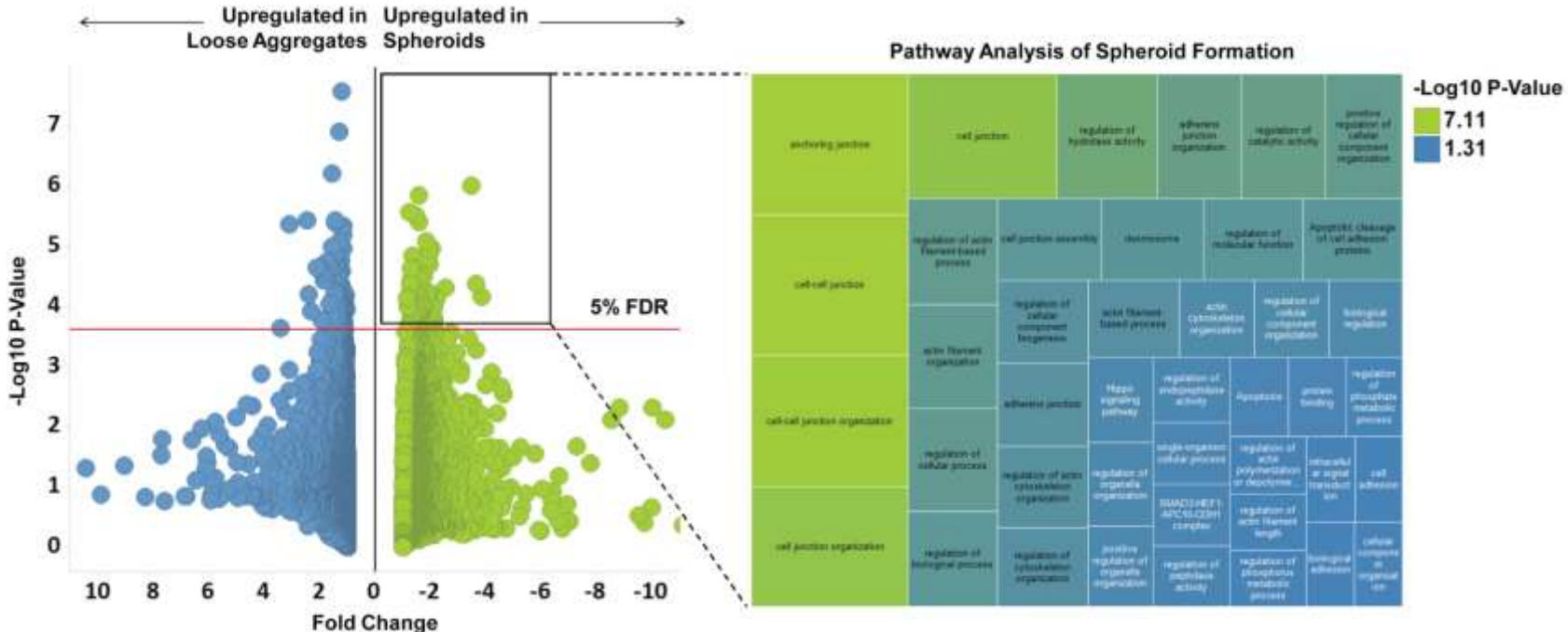


Figure 4. Genomic traits necessary for spheroid formation. (D) Individual genes associated with these biological processes are listed

(D)

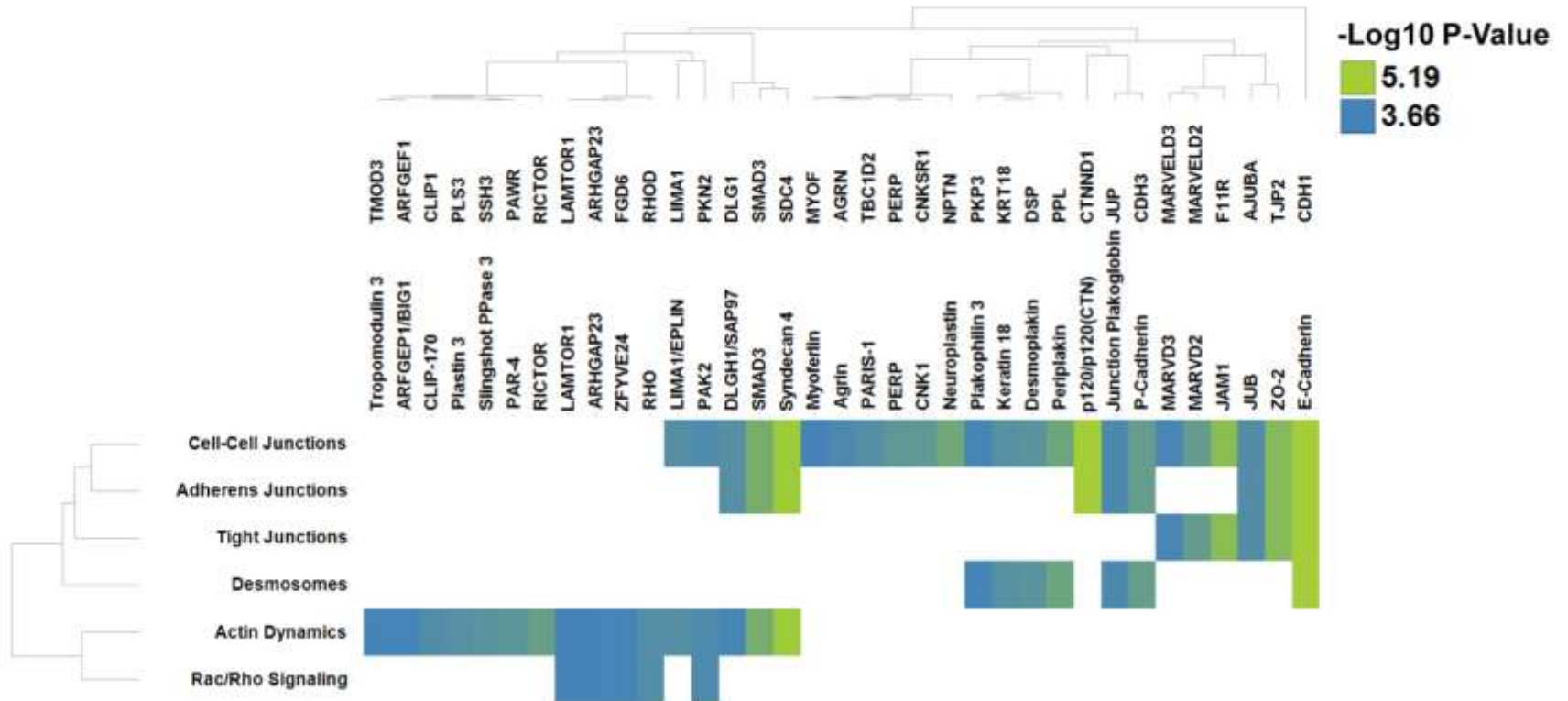




Figure 4. Genomic traits necessary for spheroid formation (E) Two-thirds of the most significantly differentially expressed genes fall into one of these two biological pathways. (F) Schematic of cell-cell junctions most highly represented.

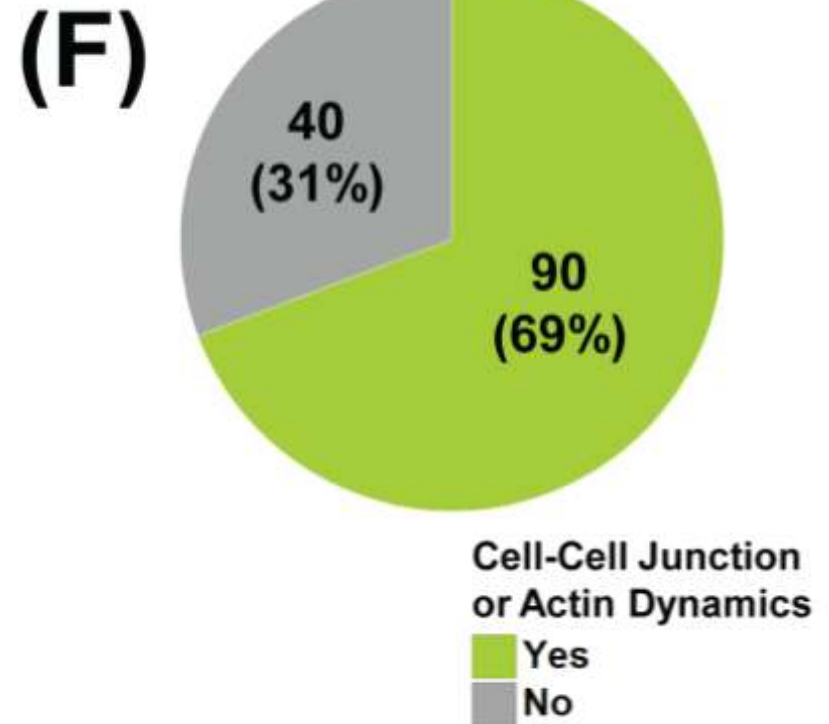
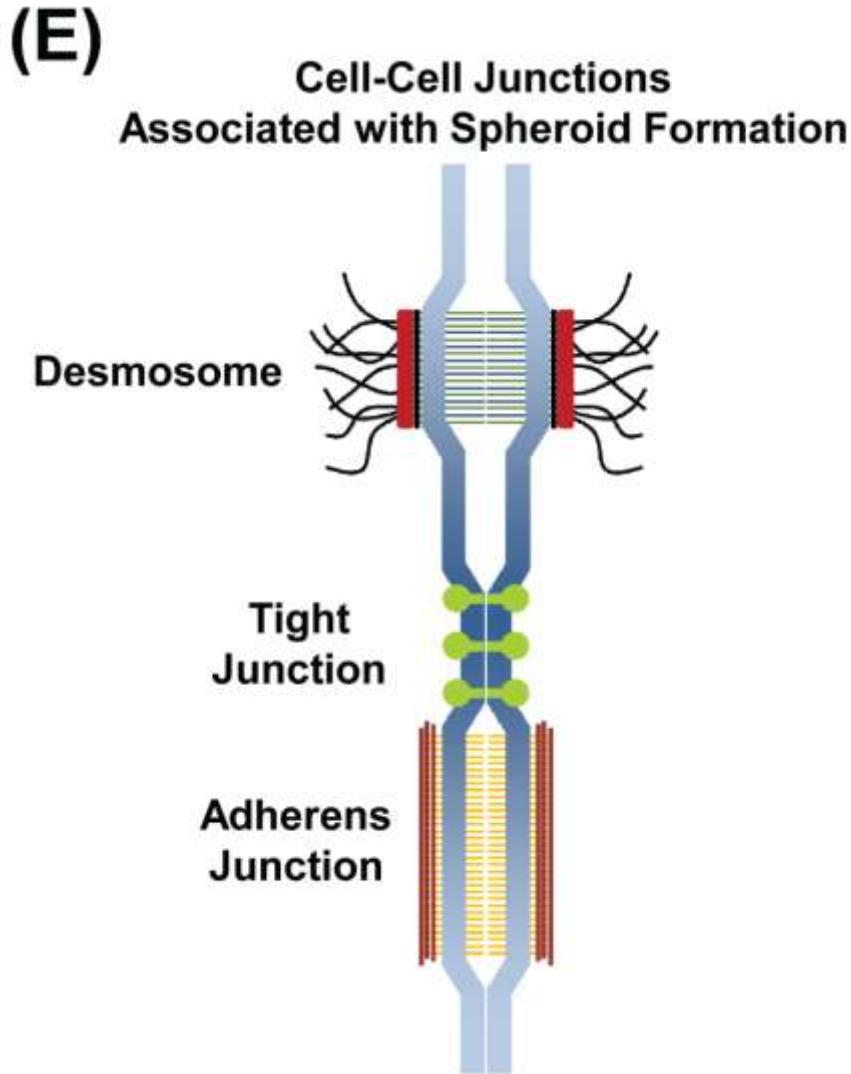


Figure 4. Genomic traits necessary for spheroid formation. (G) Mutation analysis identifies lesions associated with the inability to form spheroids.

(G)

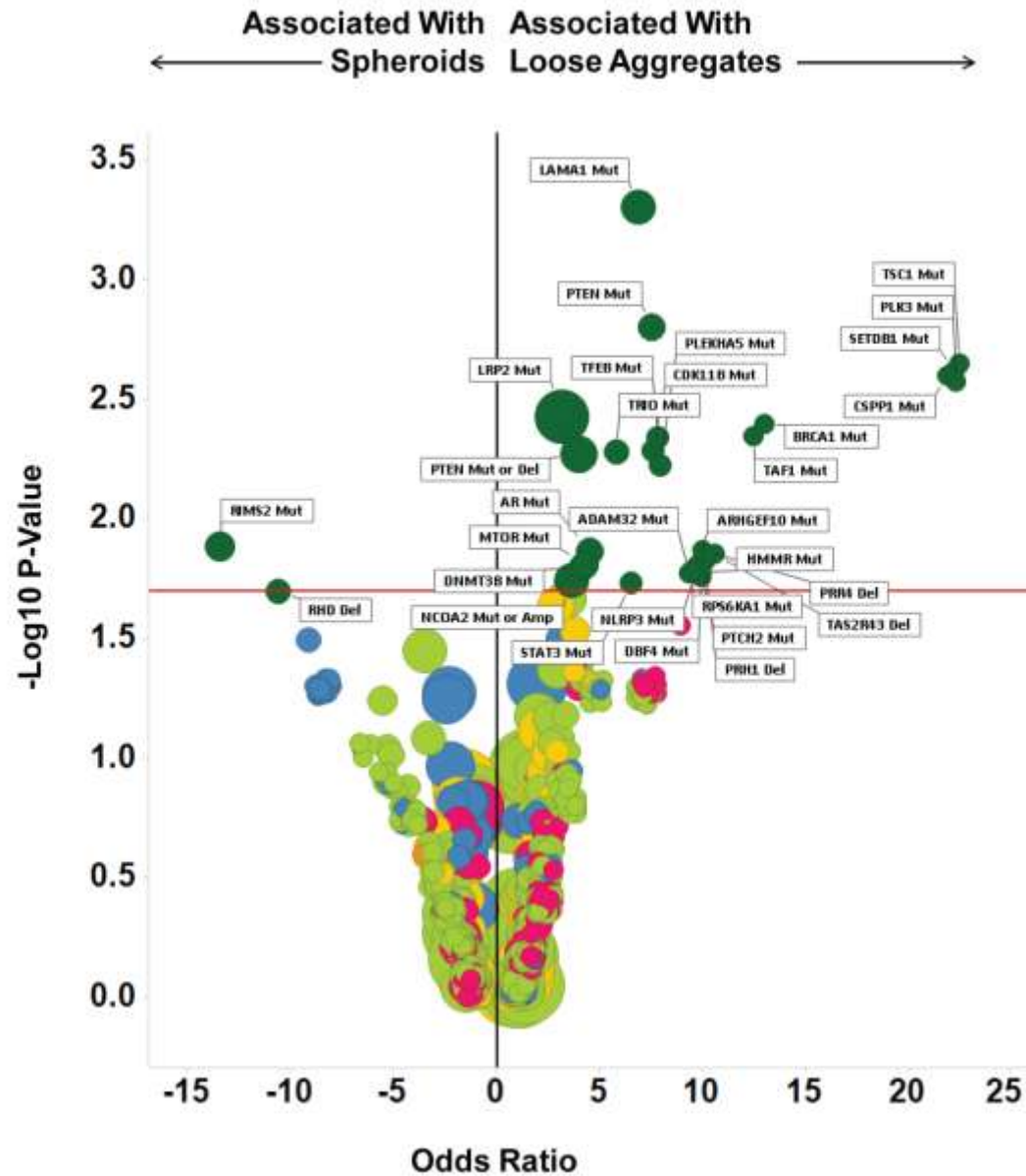
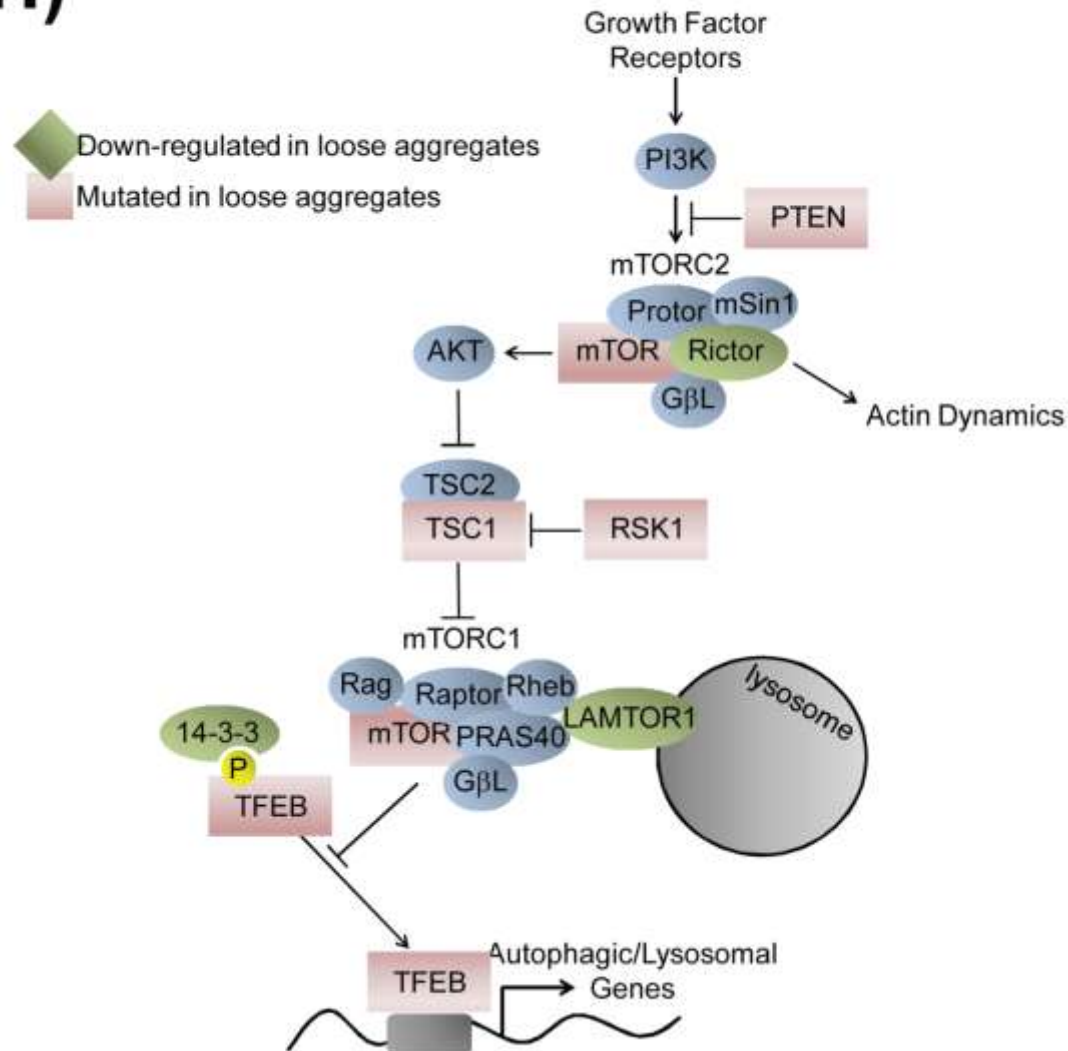


Figure 4. Genomic traits necessary for spheroid formation. (H) Enrichment of lesions within the mTOR pathway is associated with a failure to form spheroids in culture.

**(H) mTOR Pathway Dysregulation Associated With Loss of Cell-Cell Junctions**



## SUMMARY

- OncoPanel™-3D is a collection of 100+ cancer cell lines suitable for drug response profiling under 3D cell culture conditions that phenocopy in vivo tumors with respect to morphology and the presence of a hypoxic core.
- The time required for spheroid formation varies by cell line and is correlated with doubling time in 2D culture.
- Drug responses measured by high-content imaging are similar to responses measured by metabolic activity assays, such as CellTiterGLO-3D.
- Drug response in 2D vs. 3D varies by cell line.
- Genomic biomarkers measured in 2D culture predict a cell line's capacity for spheroid formation.
- The upregulation of genes involved in cell-cell junctions including adherens junctions, tight junctions, and desmosomes, as well as genes involved in actin dynamics, are necessary for spheroid formation in 3D culture.
- Cancer cell lines that fail to form spheroids show a down-regulation of genes associated with cell-cell junctions and actin dynamics, and also display dysregulation of the mTOR pathway.