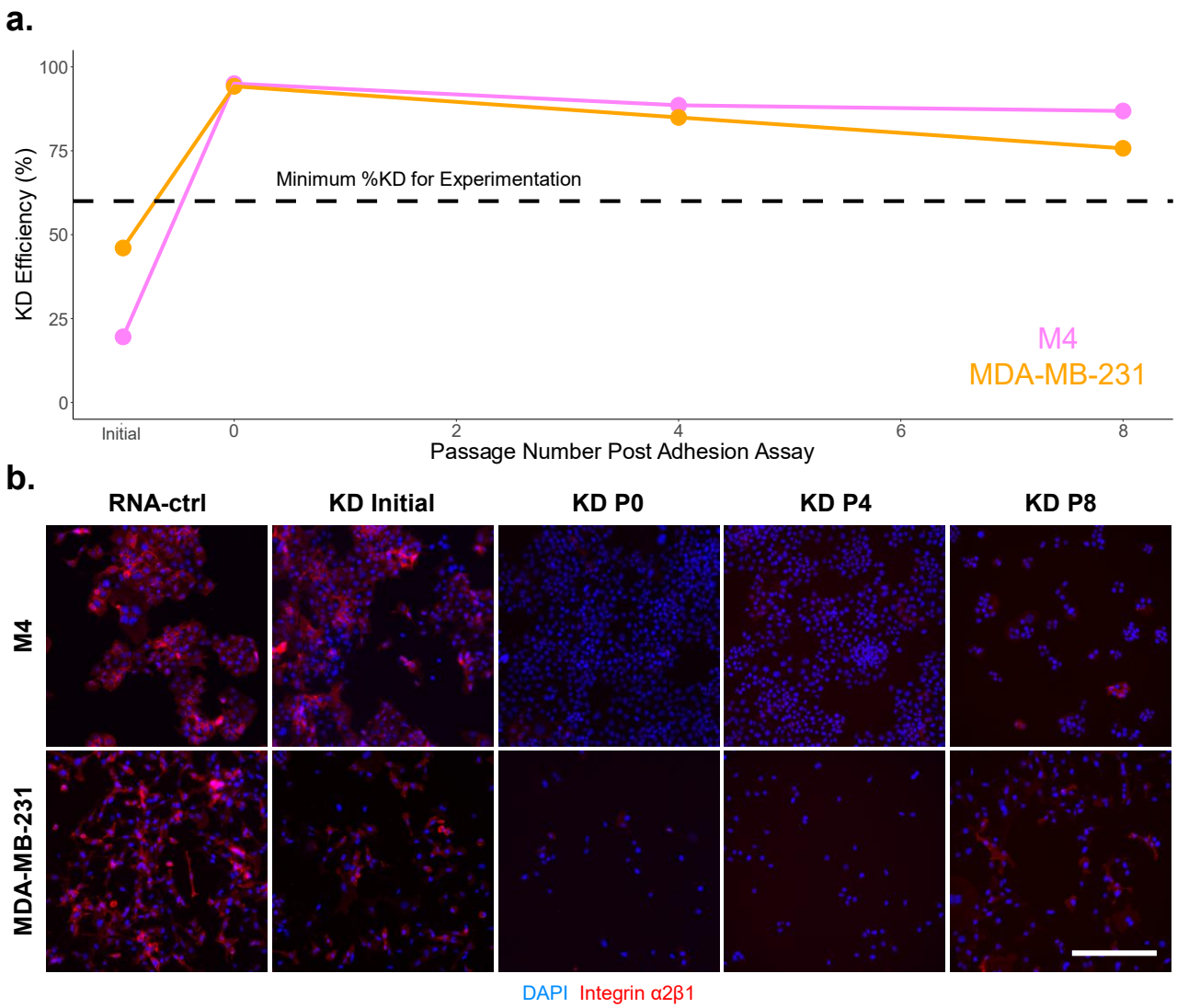
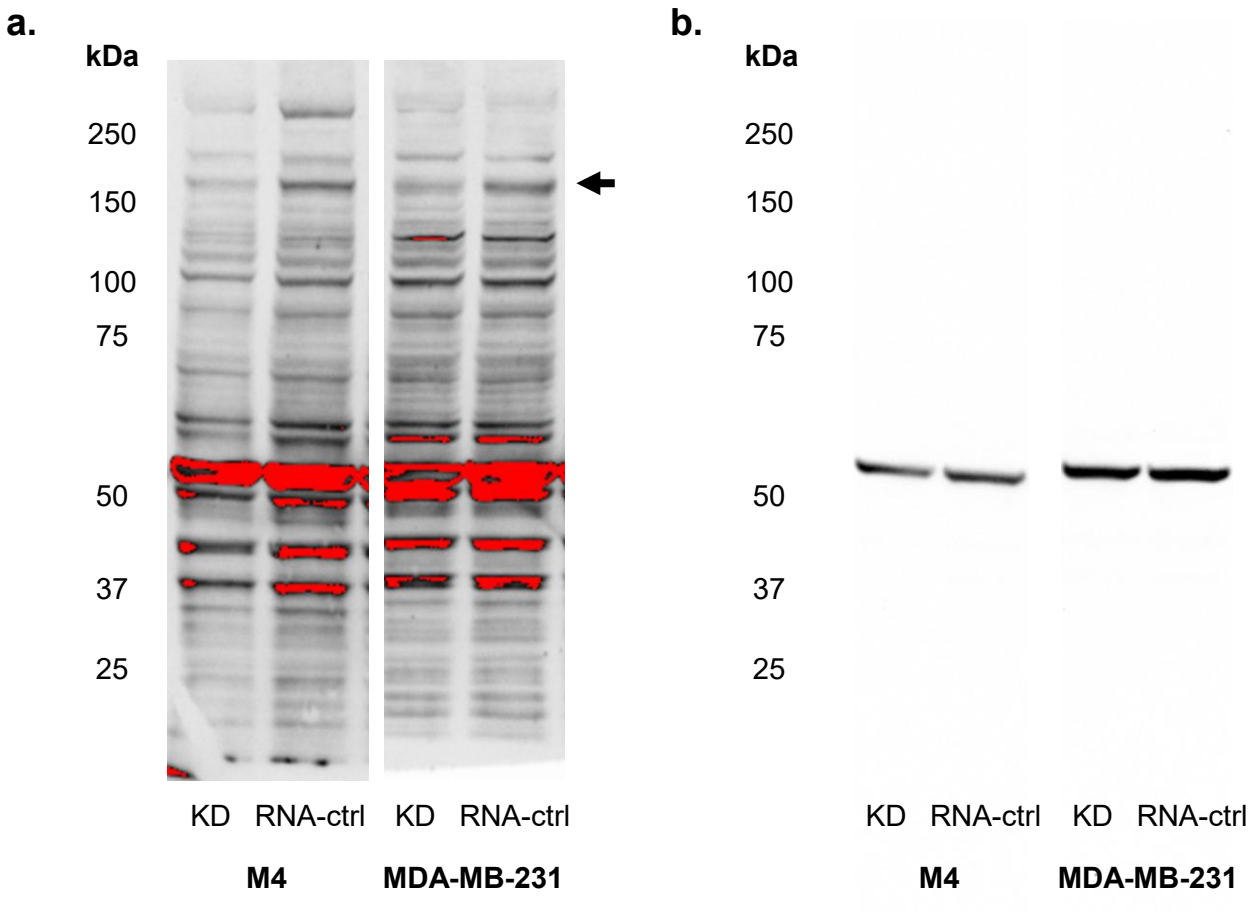
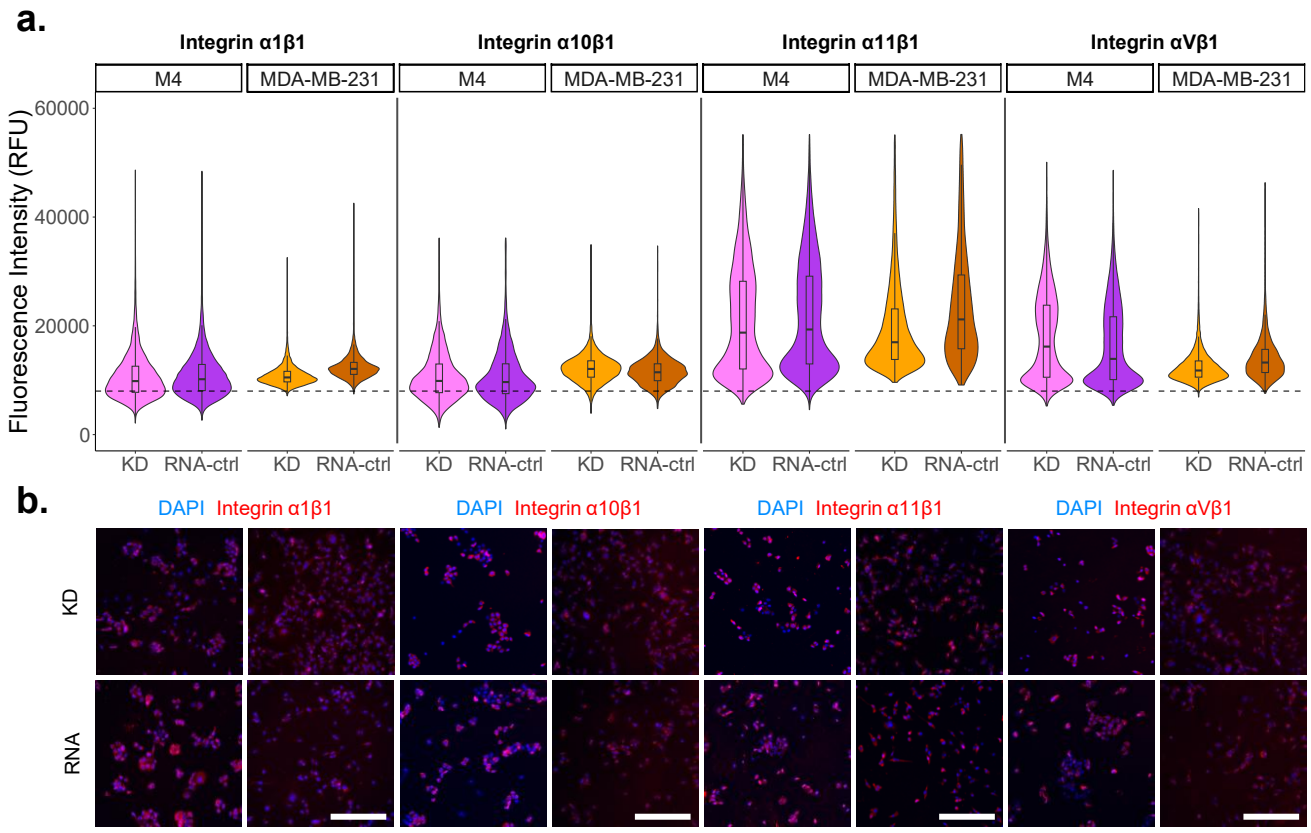


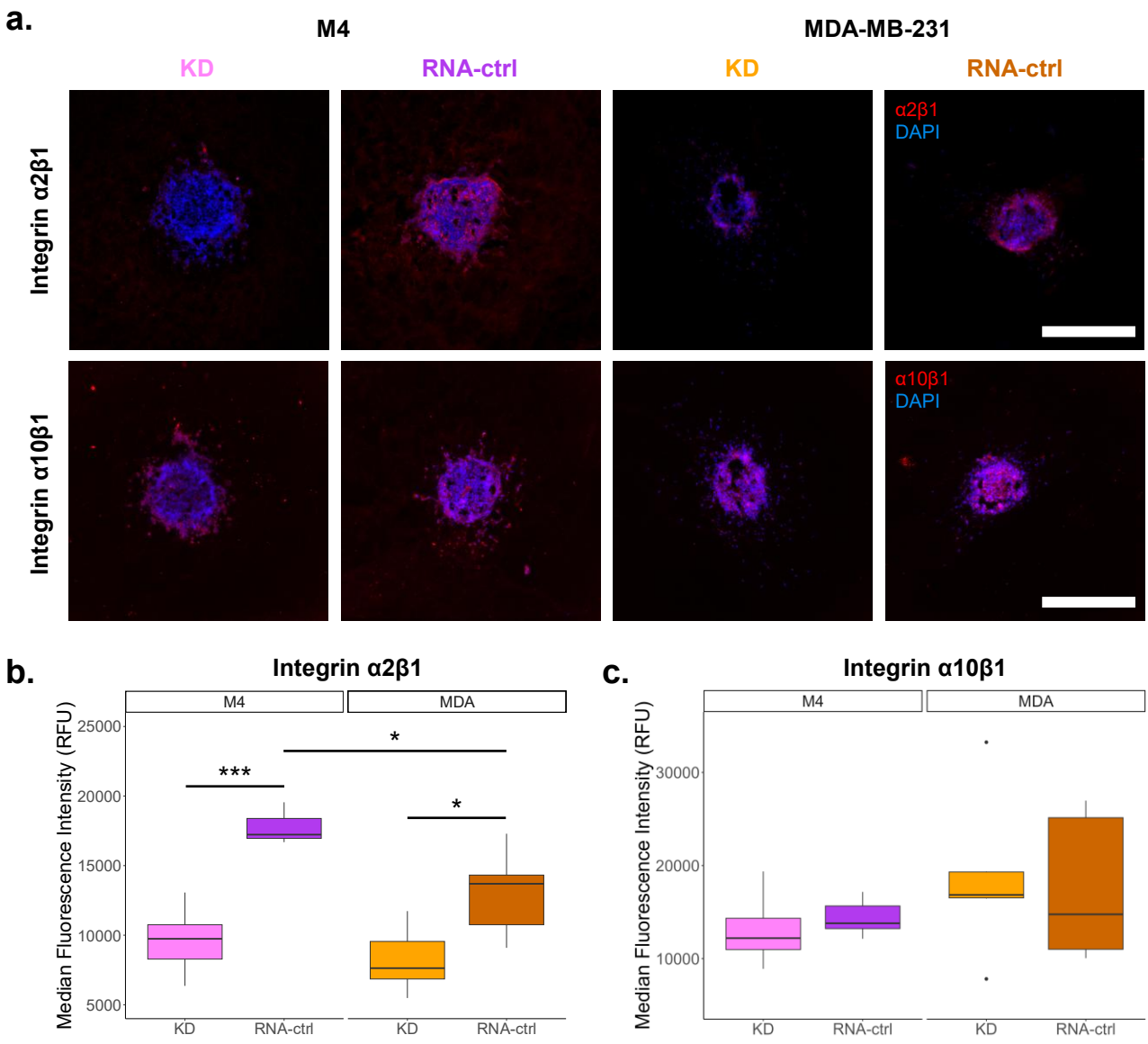
Supplementary Figure 1. Adhesion assays were essential for generating a knockdown population. Prior to adhesion assays, initial KD efficiencies for both cell lines were below the 60% KD threshold required for further experimentation. After adhesion assays, the KD populations were isolated in both cell lines reaching ~ 95% KD. A time course fluorescent labeling assay was completed, surveying the cells every 4 passages after the adhesion assay (approximately every two weeks) where the levels remained over 60% with a slight decrease over the month's time. (a) KD efficiency over time in reference to the RNA-ctrl cell line. The dotted black line represents the minimum KD efficiency needed for experimentation. (b) Representative fluorescent images in which the KD efficiency was calculated. Scale bar = 200 μm.



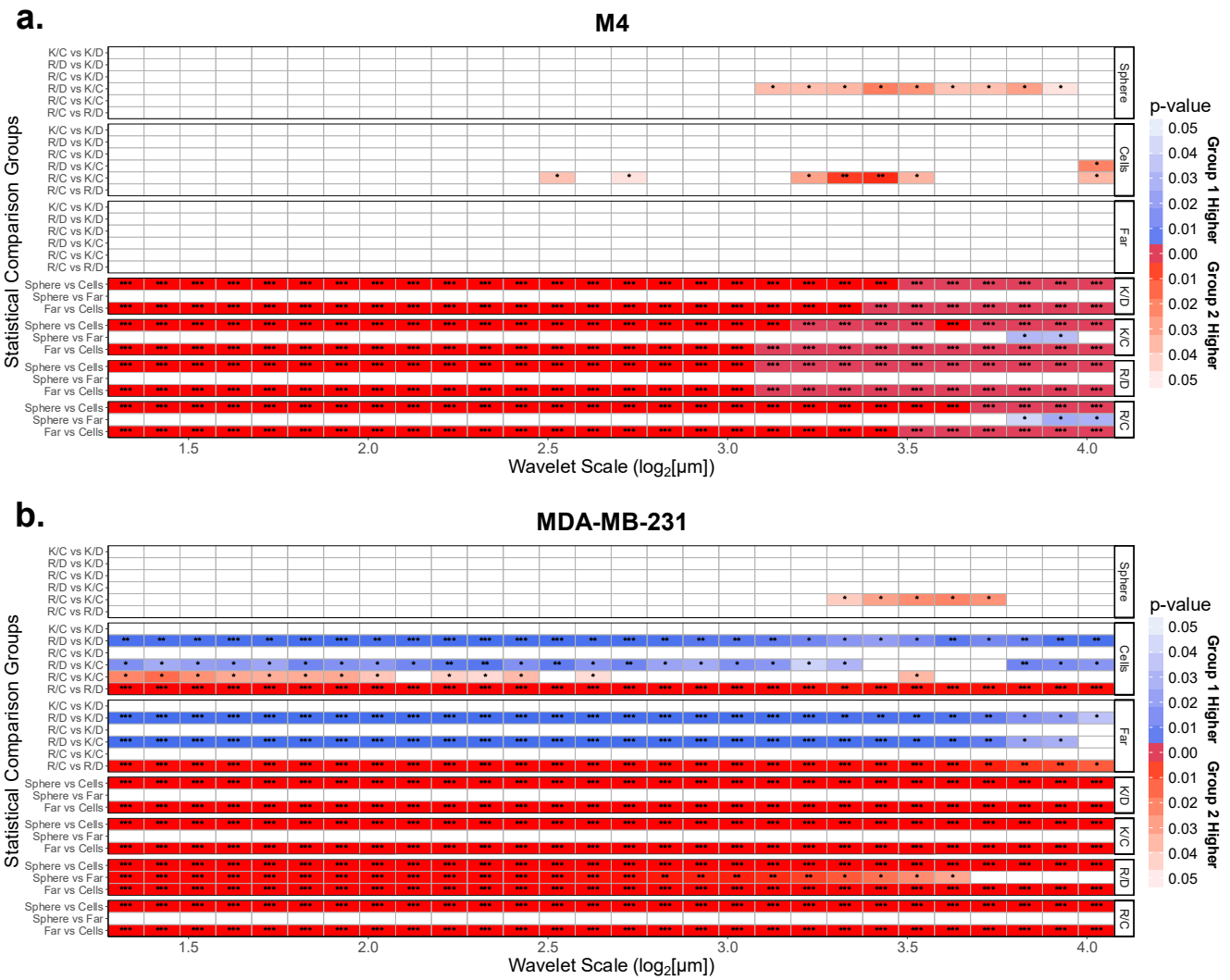
Supplementary Figure 2. Uncropped Western blot. (a) Integrin α2β1 and (b) β-tubulin uncropped Western blots. The black arrow represents the region that was cropped in the original figure. Red regions indicate areas of oversaturation.



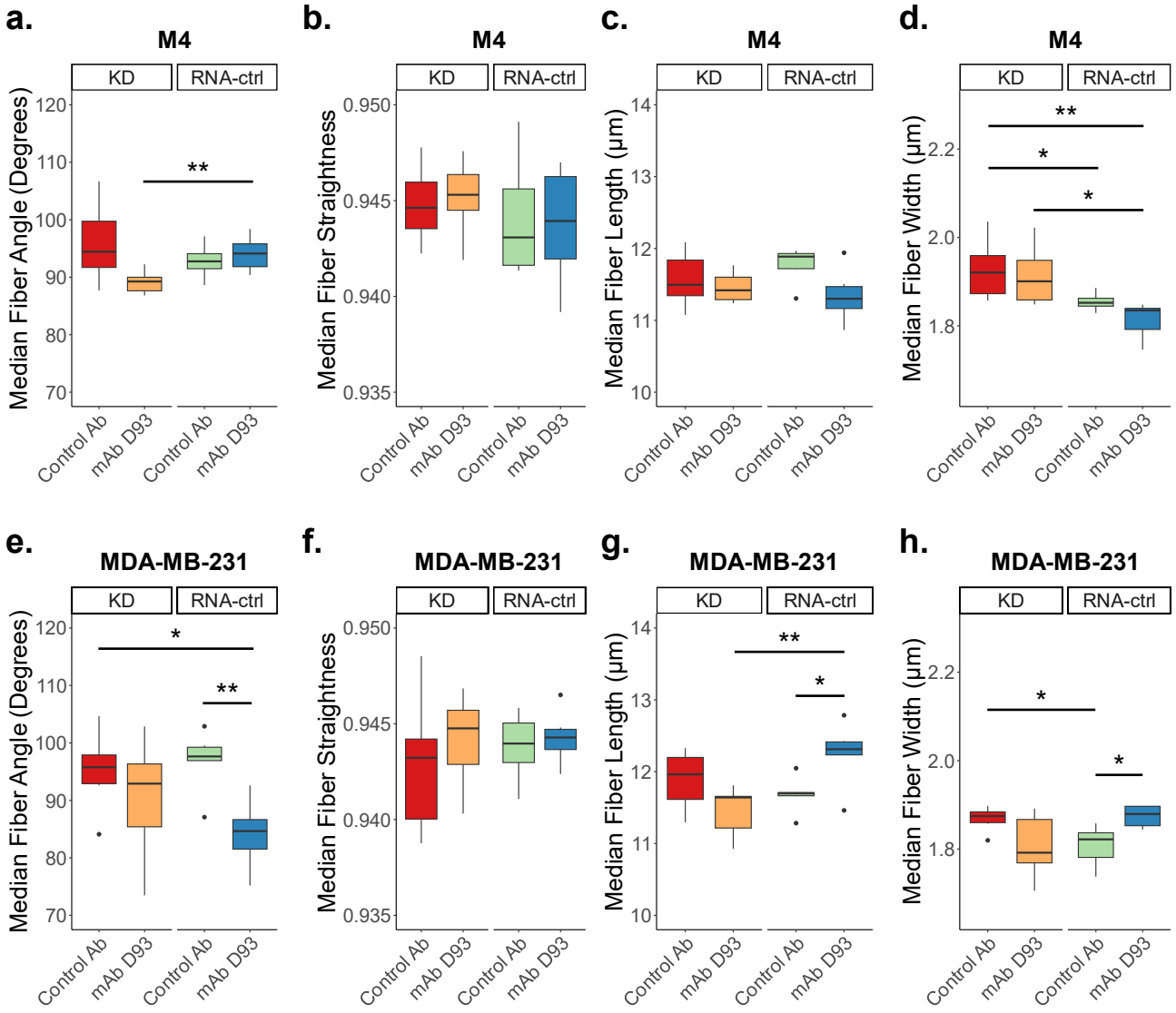
Supplementary Figure 3. Knockdown of integrin α2β1 did not impact the other collagen-binding integrins. No differences in integrin expression levels between the RNA-ctrl and KD cell lines were seen in the other collagen binding integrins (α1β1, α10β1, and α11β1) as well as the fibronectin integrin αVβ1 following integrin α2β1 KD. (a) Relative fluorescent intensity of the integrin signal. The dotted black line represents the background fluorescent intensity. (b) Representative fluorescent images of each integrin. Scale bars = 200 μm.



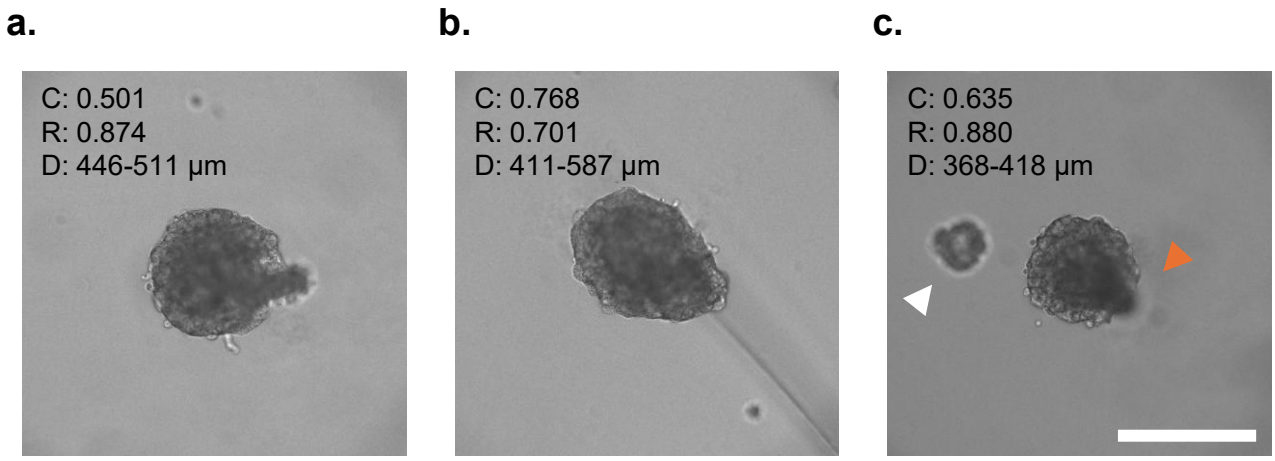
Supplementary Figure 4. Ensuring 3D culture does not impact key integrin levels observed in 2D culture. (a) Spheroids were sliced and fluorescently labeled for integrins α2β1 and α10β1. Scale bars = 500 μm. Statistical analysis revealed (b) integrin α2β1 KD is still observed in both cell lines and (c) no upregulation of integrin α10β1 is present.



Supplementary Figure 5. 2D WTMM statistical analysis. Statistical analysis for the 2D WTMM methodology on the forward SHG images for the (a) M4 and (b) MDA-MB-231 cell lines. The p-values are color coded blue when the first statistical comparison group has a higher anisotropy value and red for the inverse. White indicates no statistical difference. The categories listed on the right indicate the region or cell line where the statistical analysis was performed. K = KD, R = RNA-ctrl, C = Control Ab, D = mAb D93. N = 6 for all groups.

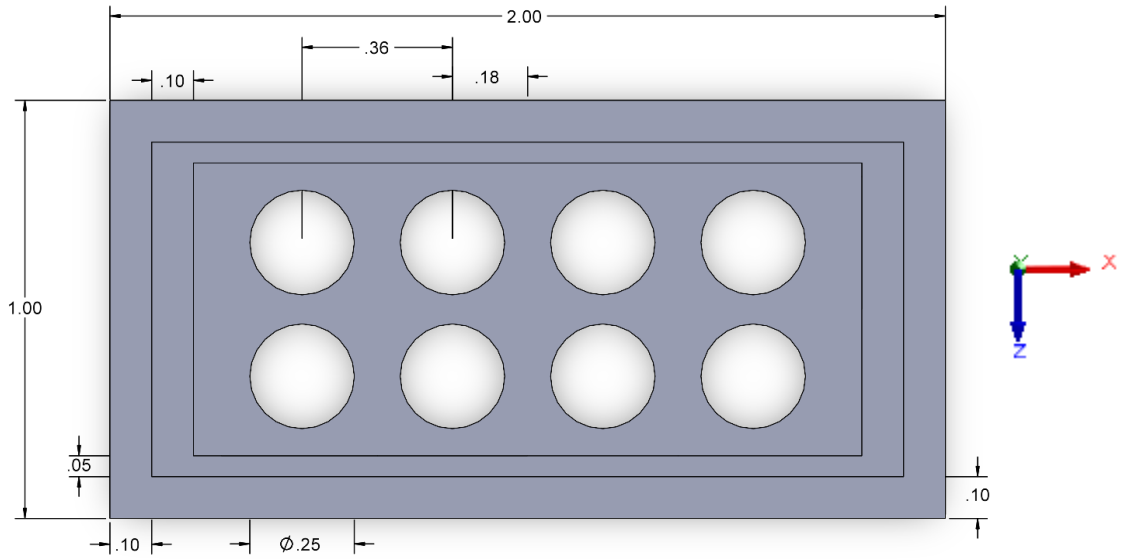


Supplementary Figure 6. CT-FIRE analysis of the far region. Individual collagen fiber (a,e) angle, (b,f) straightness, (c,g) length, and (d,h) width were calculated using CT-FIRE of both the (a-d) M4 and (e-h) MDA-MB-231 cell lines. N = 6 per group.

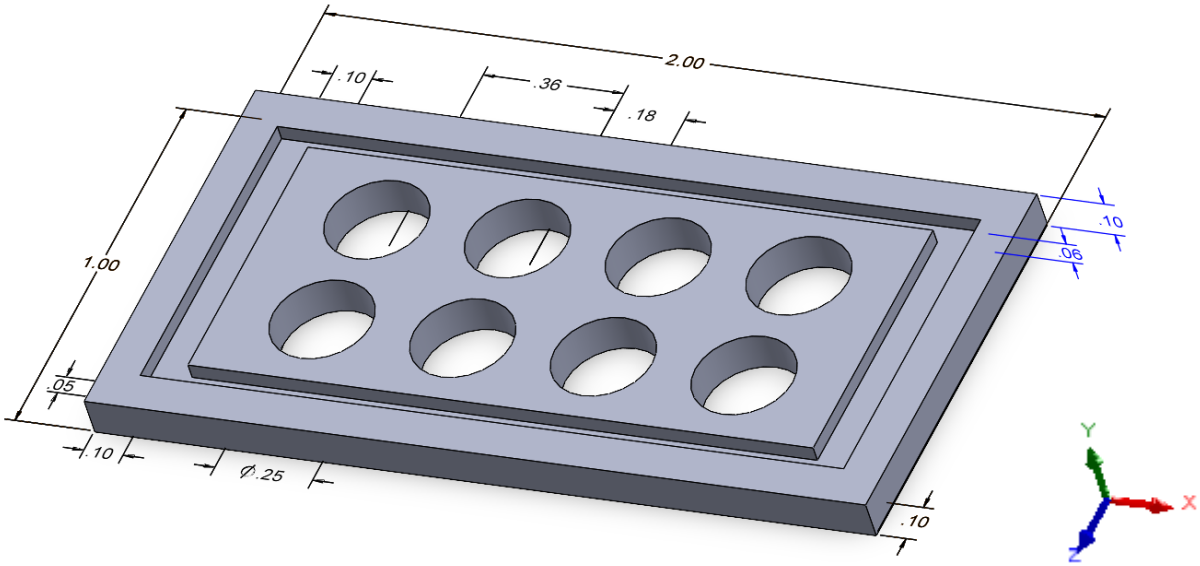


Supplementary Figure 7. Circularity, roundness, diameter, and visual inspection are required for successful spheroid screening. Widefield brightfield images were used for spheroid screening prior to further spheroid experimentation. The spheroids were analyzed regarding their circularity (C > 0.55), roundness (R > 0.80), diameter (350 < D < 450 µm), and on visual appearance. Circularity incudes the perimeter whereas roundness includes the major axis. (a) Spheroids with small protrusions that did not impact the overall shape passed on roundness but failed on circularity. (b) Whereas spheroids with a more elliptical shape passed on circularity but failed on roundness. (c) However, visual inspection was still required to catch secondary spheroids in the same well (white arrow) as well as out of focus protrusions (orange arrow). Scale bar = 500 µm.

a.



b.



Supplementary Figure 8. 3D printed holder for two photon imaging. (a) Top view and (b) rotated side view of the 3D printed holder used to hold the collagen hydrogel spheroid models after 72-hour migration experiments and fixation. A 0.4-inch-deep channel runs around the wells to catch any overflow mounting solution. The 3D holder was printed with 0.4 mm black PLA with a normal (0.15 mm) resolution and 20% infill density. The holder was glued to a glass microscope slide with Loctite 401 adhesive prior to sample loading. Dimensions in inches.