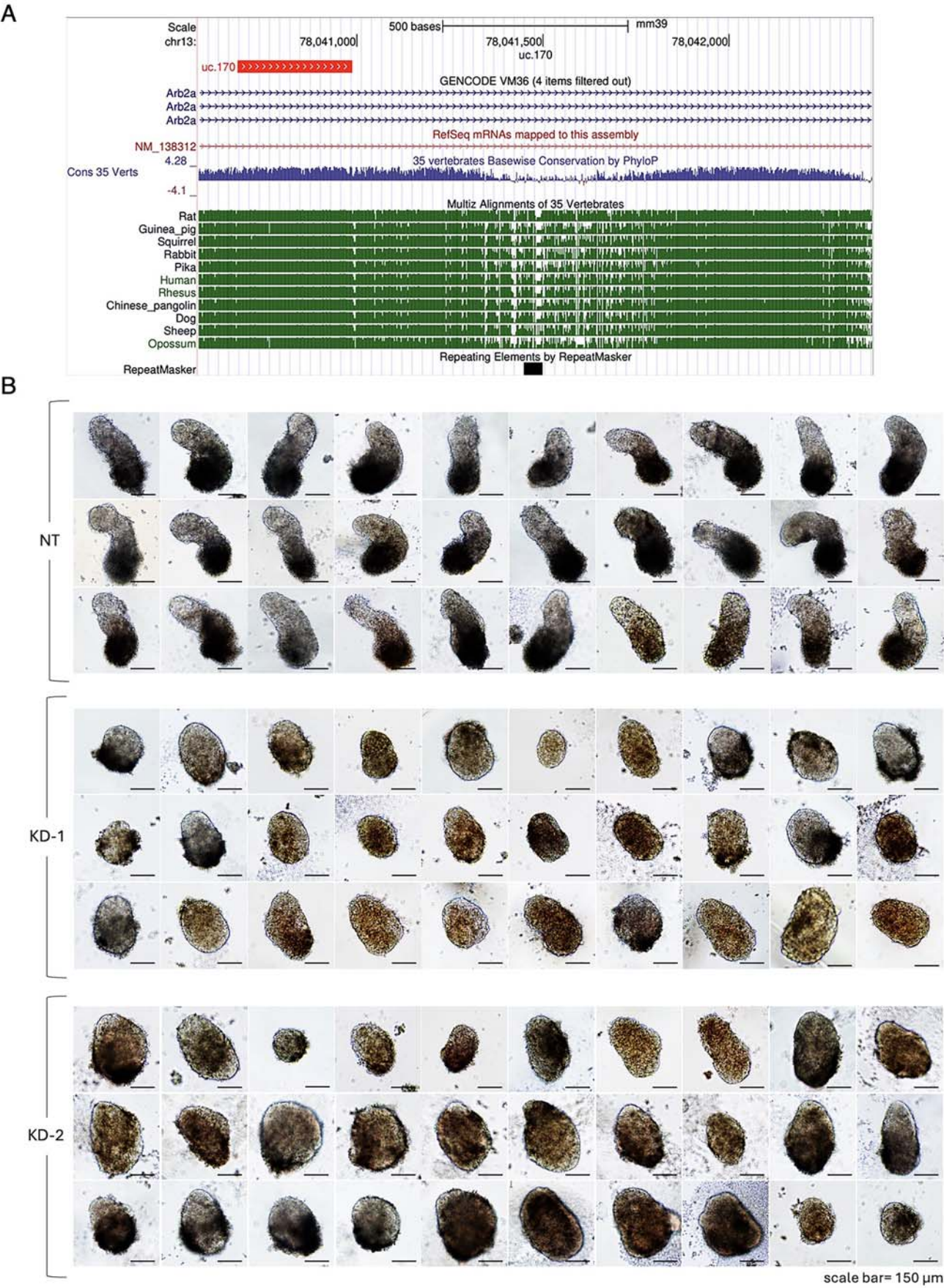


Expanded View Figures

Figure EV1. *T-UCstem1* conservation across species and shape heterogeneity showed by *T-UCstem1* KD aggregates.

(A) In red [NM_1138112](#) is *T-UCstem1*, while uc.170 is the ultra-conserved sequence by Bejerano et al, [2004](#) Cons 35 verte is the phyloP plot, with sites predicted to be conserved are assigned positive scores (shown in blue), while sites predicted to be fast evolving are assigned negative scores (shown in red). Multiz Alignments of 35 Vertebrates depicts pairwise alignments of some species to the mouse genome indicating alignment quality. (B) The figure showing the heterogeneity of *T-UCstem1* KD gastruloids compared to Control (NT). Scale bar, 150 μ m ($n = 4$ independent experiments; 30 gastruloids/condition).



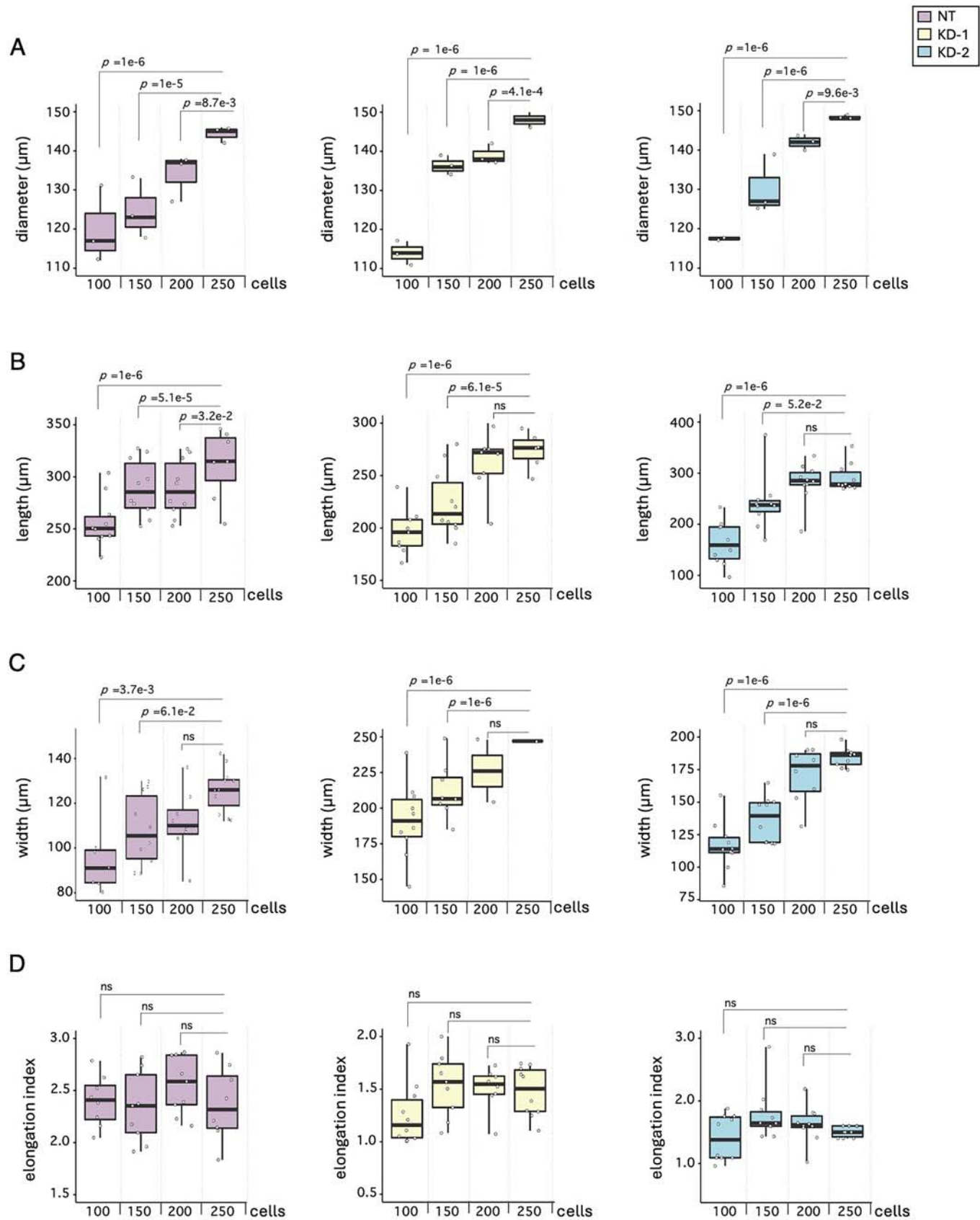


Figure EV2. *T-UCstem1* KD effect is independent on the initial cell number.

(A) Boxplot diagrams of the aggregate diameter at 48 h of NT (left), KD-1 (middle) and KD-2 (right). Boxplots display the minimum, first quartile, median, third quartile, and maximum. Statistical significance was assessed by one-way ANOVA with Tukey's multiple comparison test. *P* values of ≤ 0.05 were considered statistically significant. (B–D) Boxplot diagrams of the gastruloids length (B), width (C) and elongation index (D) at 120 h of NT (left), *T-UCstem1* KD-1 (middle) and *T-UCstem1* KD-2 (right). Data are shown as mean $\pm$ SD ($n = 2$ independent experiments; 10 gastruloids/condition). Boxplots display the minimum, first quartile, median, third quartile, and maximum. Statistical significance was assessed by one-way ANOVA with Tukey's multiple comparison test. *P* values of ≤ 0.05 were considered statistically significant.

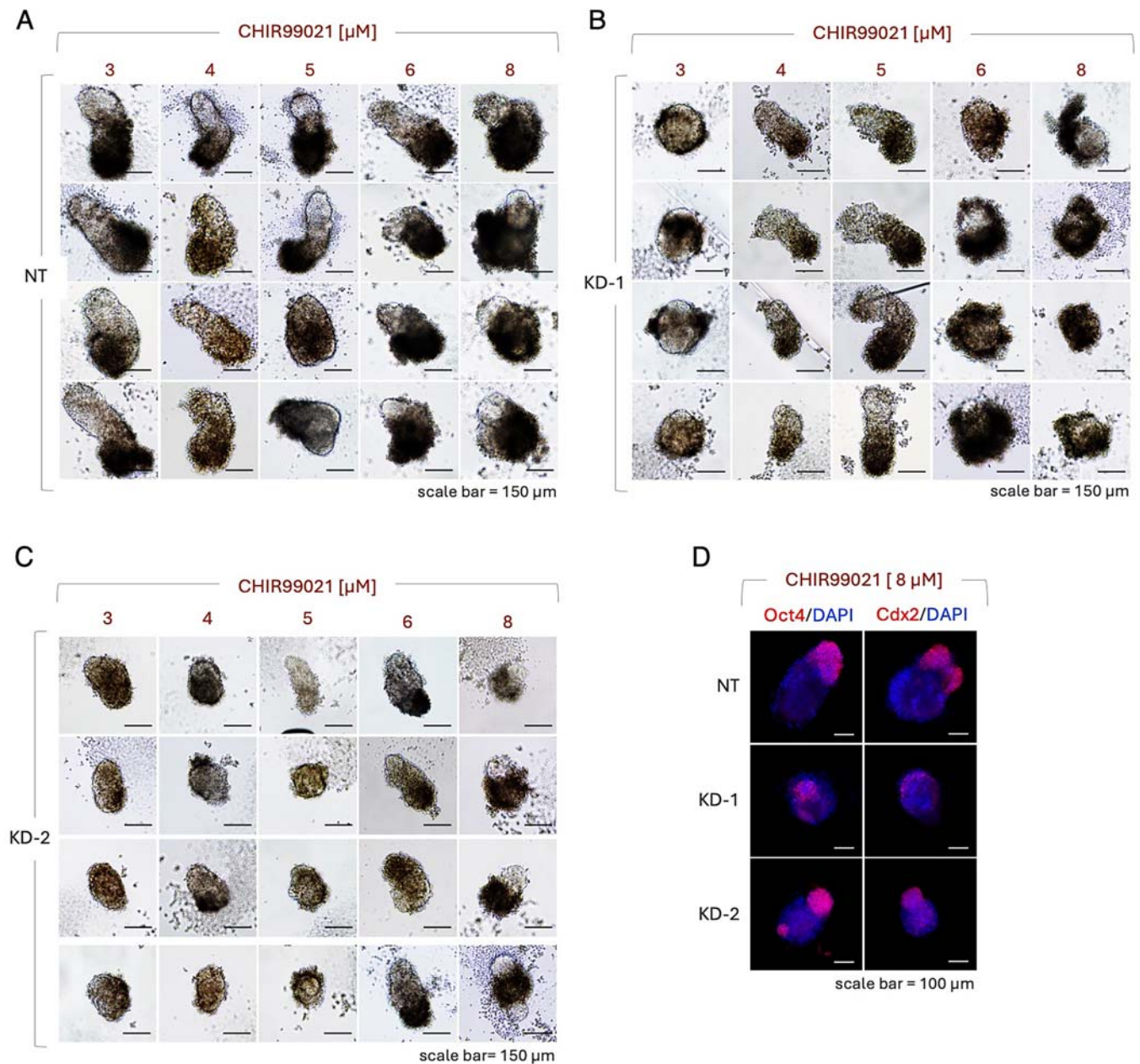
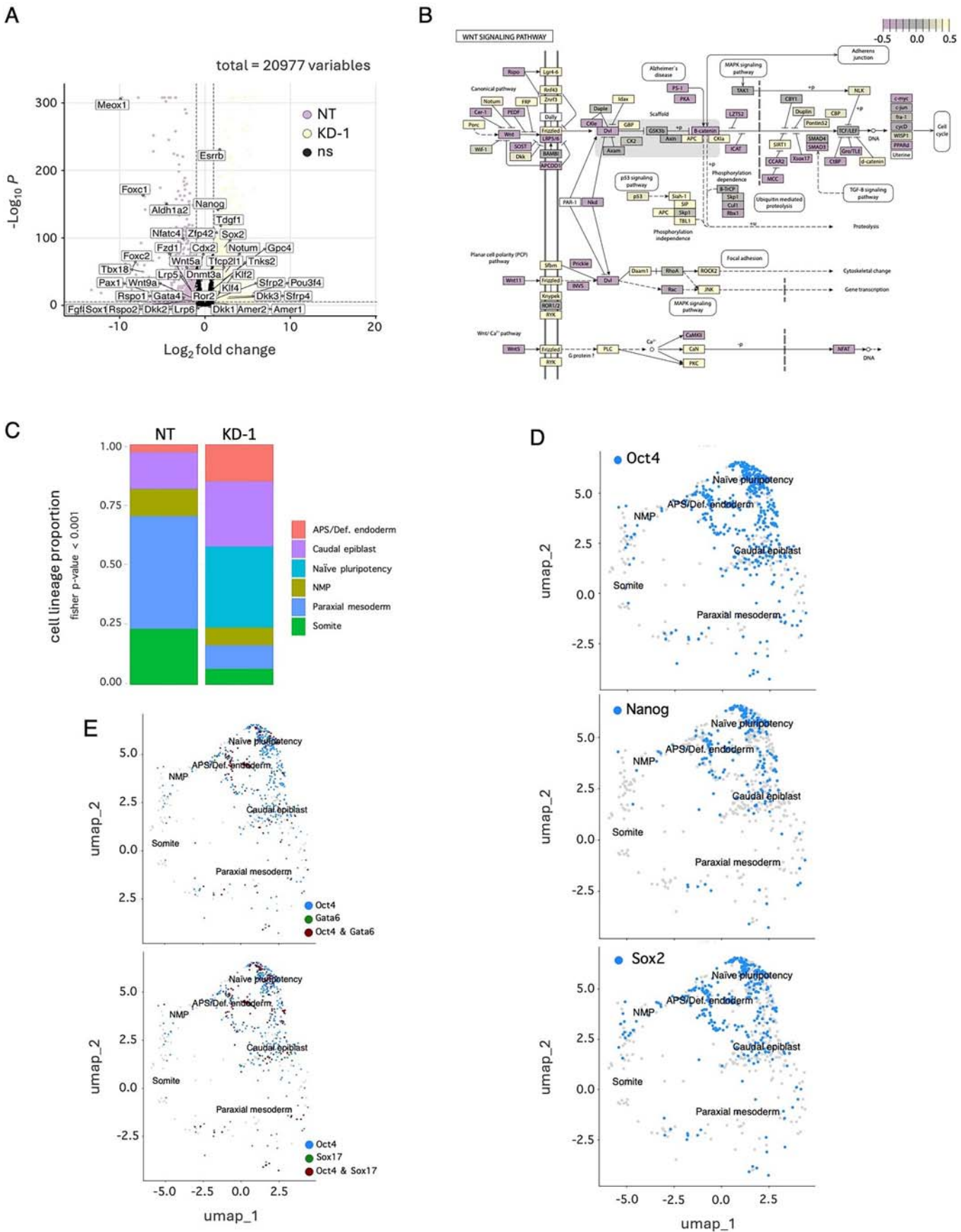


Figure EV3. Dose-dependent effect of CHIR99021 on gastruloid development.

(A–C) Representative brightfield images of Control (NT) (A), KD-1 (B), KD-2 (C) gastruloids treated with increasing concentration of CHIR99021 (3–8 μM). Scale bar, 150 μm . (D) Representative confocal images of Oct4 (pluripotency marker) and Cdx2 (differentiation marker) in gastruloids treated with 8 μM of CHIR99021. Nuclei were counterstained with DAPI (blue). Scale bar, 100 μm . All images are representative of 4 individual gastruloids.



**Figure EV4. Diverse cellular composition in *T-UCstem1* KD gastruloids versus the Control.**

(A) Volcano plot of differentially expressed genes between *T-UCstem1* KD-1 and NT gastruloids. Differential expression analysis was performed using DESeq2 (Wald test; $n = 3/\text{group}$). Genes with adjusted $p < 0.05$ and $|\log_2 \text{fold change}| > 1$ are color-coded. (B) Reference map of the WNT Signaling pathway from the KEGG pathway database; annotated features are coloured based on differential expression between KD and Control (NT). (C) Stacked barplots of the cell lineage proportion in *T-UCstem1* KD-1 and NT gastruloids. (D) UMAP plot highlighting expression of *Oct4*, *Nanog* and *Sox2* in *T-UCstem1* KD-1 gastruloids. (E) UMAP plot highlighting expression of *Oct4*, *Gata6* and *Sox17* in *T-UCstem1* KD-1 gastruloids.

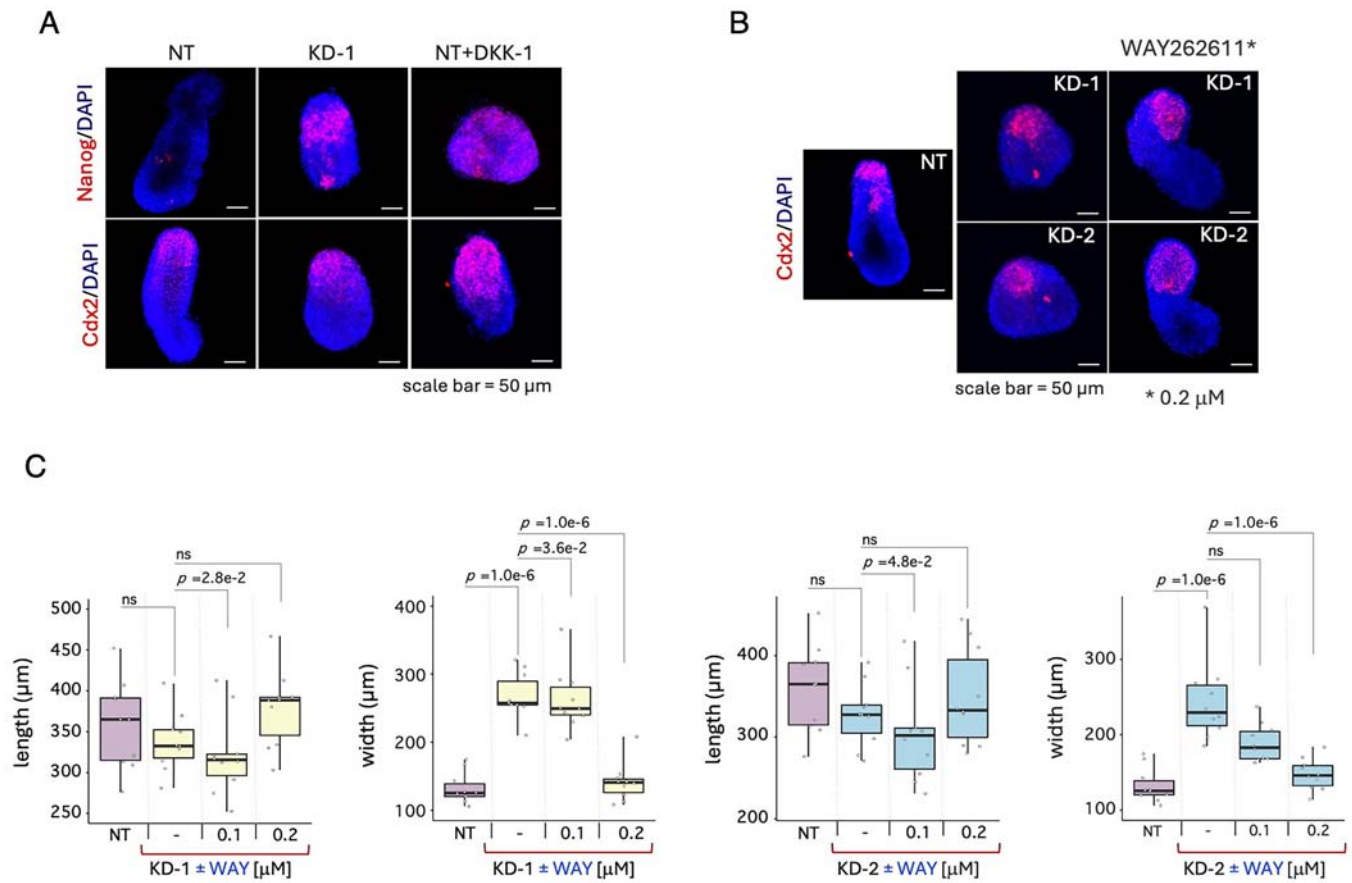


Figure EV5. DKK-1 blocks the correct gastruloid development.

(A) Representative confocal images of Nanog (pluripotency marker) and Cdx2 (differentiation marker) in gastruloids $\pm$ DKK-1 recombinant protein. Nuclei were counterstained with DAPI (blue). Scale bar, 100 μ m. All images are representative of 4 individual gastruloids. (B) Representative confocal images of Cdx2 (differentiation marker) in gastruloids NT and *T-UCstem1* KD $\pm$ WAY262611 at 0.2 μ M. Nuclei were counterstained with DAPI (blue). Scale bar, 50 μ m. All images are representative of 4 individual gastruloids. (C) Boxplot diagrams of gastruloids length (left), width (right) $\pm$ WAY262611 at 120 h. Data are shown as mean $\pm$ SD ($n = 3$ independent experiments, 10 gastruloids/condition). Boxplots display the minimum, first quartile, median, third quartile, and maximum. Statistical significance was assessed by one-way ANOVA with Tukey's multiple comparison test. P values of ≤ 0.05 were considered statistically significant.

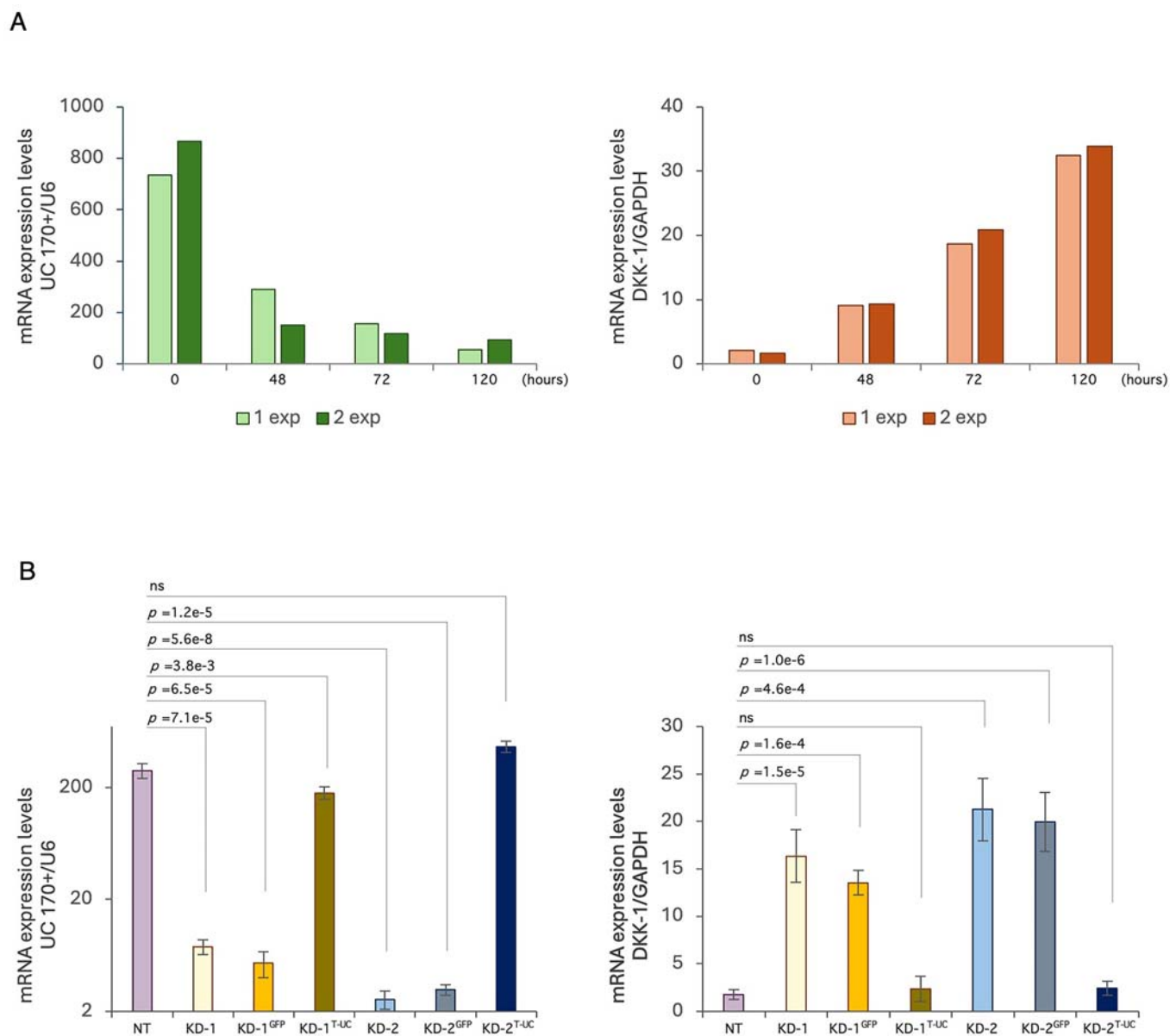


Figure EV6. Expression analysis of *T-UCstem1* and *DKK-1* in mESCs and during gastruloid development.

(A) qRT-PCR analysis of *T-UCstem1* (left) and *DKK-1* (right) expression levels at different stages of gastruloid development in control (NT) conditions. *T-UCstem1* levels were normalized to *U6*, and *DKK-1* levels to *Gapdh*. Individual values of two biological replicates are shown (Exp 1 and 2). (B) qRT-PCR analysis of *T-UCstem1* (left) and *DKK-1* (right) expression levels in control (NT), *T-UCstem1* knockdown (KD), KD^{GFP}, and KD^{T-UC} mESCs. *T-UCstem1* expression was normalized to *U6*, and *DKK-1* expression to *Gapdh* ($n = 2$ independent experiments). Data are shown as mean $\pm$ SD ($n = 3$ independent experiments). Statistical significance was assessed by one-way ANOVA with Tukey's multiple comparison test. P values of ≤ 0.05 were considered statistically significant. Source data are available online for this figure.