Day 4 gastruloids from 2 plates were collected (approximately 45-48 gastruloids, per treatment, per biological replicate) (N, biological replicate = 4) into ice cold PBS in a clean glass petri dish, washed once and transferred to another glass petri dish on ice containing cold PBS. Gastruloids were collected, briefly centrifuged to settle them down and the PBS was completely removed. Gastruloids were snap-frozen in liquid nitrogen. To count the number of cells in each gastruloid, 5 gastruloids from each batch and each treatment, were collected, trypsinised for 1 min in 50 µl of 0.05% trypsin (25300054, ThermoFisher Scientific) and neutralised using 950 µl of warm N2B27. Cells were immediately counted using the Neubauer haemocytometer chamber. Snap-frozen gastruloids were sent to EMBL-Heidelberg, Germany, for the metabolomics analysis.

Reagents:

LC-MS grade water, acetonitrile and methanol were obtained from Th. Geyer (Germany). High-purity ammonium acetate, ammonium hydroxide, and formic acid were purchased from Merck (Germany). Stable isotope labelled amino acids (MSK-MET1-1; Cambridge Isotope Laboratories, MA, USA) were used as internal standards for untargeted metabolomics.

Sample preparation:

Metabolite extraction was performed by addition of 200 µL 80 % methanol (including 2 % (v/v) internal standards) and subsequent homogenization on dry ice via a bead beater (FastPrep-24; MP Biomedicals, CA, USA) at 6.0 m/s (5 x 30 s, 5 min pause time) using 1.0 mm zirconia/glass beads (Biospec Products, OK, USA). After centrifugation for 10 min at 15,000*g* and 4 °C with a 5415R microcentrifuge (Eppendorf, Hamburg, Germany), supernatants were transferred and the remaining sample residues were reextracted with 200 µL acetonitrile:methanol:water (2:2:1, v/v) containing 1 % (v/v) formic acid using identical settings for homogenization and centrifugation. Corresponding supernatants of both extraction steps were combined and dried under a stream of nitrogen. Dried samples were reconstituted in 60 µL acetonitrile:methanol:water (2:2:1, v/v), vortexed for 5 min, centrifuged, and transferred to analytical glass vials. The LC-MS/MS analysis was initiated within one hour after the completion of the sample preparation.