Multidrop Combi (Thermo) to the compound plates, and mixed by inversion. Following a 30 min preincubation of enzyme with compound at room temperature (~22 ºC), the substrate FAM-QRVRRAVGIDK-TAMRA (SEQ ID NO:1) (5 µL of a 1 µM solution in assay buffer) was added using a Multidrop Combi to the entire assay plate. The plates were centrifuged at 500Xg for 1 minute and incubated at room temperature for two hours. Enzyme inhibition is then quantified using an Envision instrument (PerkinElmer). Data were normalized to maximal inhibition determined by 1 µM Decanoyl-Arg-Val-Lys-Arg-~~Lys~~ Chloromethylketone (SEQ ID NO:2) (Calbiochem #344930 or AnaSpec #808143).

Furin cell-based assay

This assay uses an image-based platform to evaluate the intracellular activity of compounds of the invention. Reactions were performed in black 384-well, tissue culture-treated, clear bottom plates (Greiner). Compounds under analysis were dissolved in DMSO (1.0 mM) and serially diluted 1 to 3 with DMSO through eleven dilutions. This creates a final compound concentration range from 0.00017 to 10 µM, and 0.1 µL of each concentration was transferred to the corresponding well of the assay plate. Assays were initiated by the addition of U2OS cells simultaneously transduced with a BacMam-delivered construct containing a Golgi-targeting sequence followed by a 12-amino acid furin/PCSK cleavage site from Bone Morphogenic Protein 10 (BMP10) and then GFP at the C terminus. The furin cleavage site sequence was flanked by glycine rich linkers (GalNAc-T2-GGGGS-DSTARIRRNAKG-GGGGS-GFP) (SEQ ID NO:3). Briefly, frozen cells are thawed in assay media (Dulbecco's Modified Eagles Medium Nutritional Mixture F-12 (Ham) without phenol red containing 5% FBS) and diluted to deliver 6000 cells/well (50 µL) to the plate using a Multidrop Combi (Thermo).

After a 24-hour incubation period at 37 ºC, the cells are stained with Cell Mask Deep Red, fixed in paraformaldehyde and the nuclei stained using Ho33342. The Golgi-targeted GFP forms bright punctate clusters within the cell. In the absence of a furin inhibitor, the endogenous protease cleaves GFP from its N-acetylgalactosaminyltransferase-2 Golgi tether, releasing GFP into the Golgi lumen where fluorescence is diluted below the threshold of assay sensitivity. In the presence of a cell-permeable furin inhibitor, GFP fluorescence increases as intra-Golgi protease activity is reduced. Cellular GFP intensity is determined by image-based acquisition (Incell 2200,