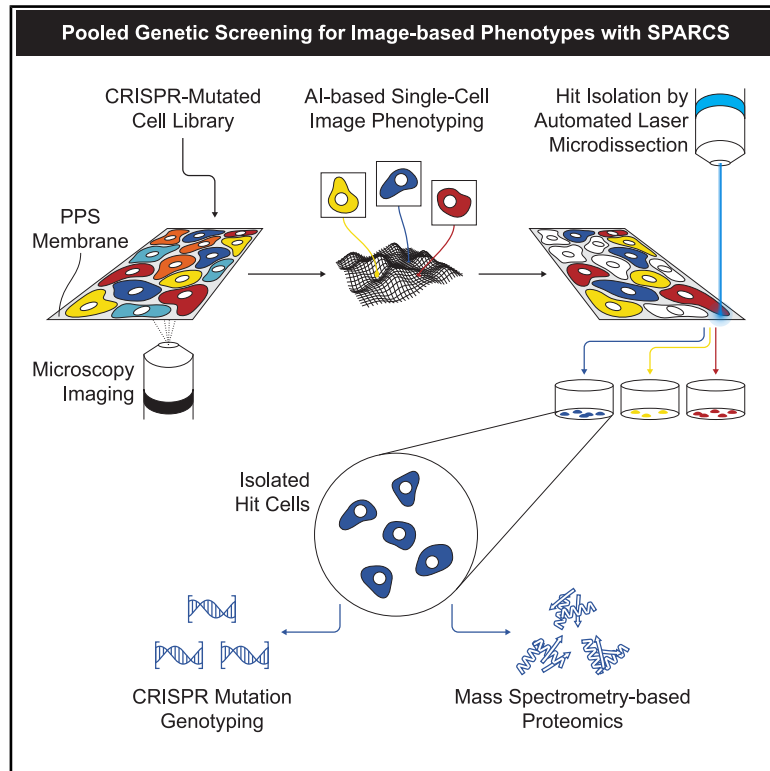


SPARCS enables scalable recovery of complex image-based phenotypes for genetic screening

Graphical abstract



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In brief

Current genetic screens face a trade-off between scaling to large mutant libraries and measuring information-rich phenotypes. SPARCS automates laser microdissection to enable pooled forward genetic screens for complex image-based phenotypes at scale. By physically recovering individual mutants, SPARCS supports multimodal profiling of hits beyond genotyping, including via mass spectrometry-based proteomics.

Highlights

- SPARCS enables image-based CRISPR screening of complex phenotypes at scale
- AI guides recovery of mutant cells *in situ* by automated laser microdissection
- Genome-wide screens in 70 million cells reveal regulators of autophagy and STING signaling
- Profiling isolated hits by mass spectrometry proteomics maps pathways driving phenotypes

Resource

SPARCS enables scalable recovery of complex image-based phenotypes for genetic screening

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SUMMARY

Forward genetic screening links genotype to phenotype by introducing random genetic perturbations and identifying phenotype-altering mutations. Although genome-scale screens are routine for simple phenotypes in cultured cells, extending them to complex image-based phenotypes remains challenging. Here, we present spatially resolved CRISPR screening (SPARCS), a microscopy-based platform for forward genetic screening on single-cell images. SPARCS physically isolates mutants *in situ* by automated laser microdissection, enabling image-based screening at unprecedented scale with multimodal hit phenotyping. We demonstrate SPARCS in genome-wide CRISPR knockout screens of autophagosome formation and activation of the immune sensor STING across 70 million cells. Via machine learning-based image analysis, SPARCS recovered most macroautophagy genes and identified GPHR as a pH-dependent regulator of STING. Mass spectrometry-based proteomics of isolated hit cells revealed endoplasmic reticulum (ER)/Golgi disruption and nominated additional STING regulators via *in silico* perturbation modeling. These results establish SPARCS as a scalable platform for genome-wide genetic screening of complex cellular phenotypes with a proteome-level readout.

INTRODUCTION

Genetic screens offer a powerful approach to dissecting the complexity inherent in biological systems. An unbiased way to map phenotypic changes to genetic changes is forward genetic screening: from a library of genetic variants generated by random mutagenesis, mutants with interesting phenotypes are selected and their genotypes determined. This approach has led to groundbreaking discoveries in a variety of model organisms.^{1–3} Recently, CRISPR-based genome editing technologies⁴ have enabled the generation of large mutant libraries in which a single gene is knocked out in each cell.⁵ Individual genetically perturbed cells can now be profiled for complex phenotypes,⁶ including their transcriptome,^{7–15} protein expression,¹⁶ spatial composition,¹⁷ tissue localization,¹⁸ and chromatin landscape^{19,20} and combinations thereof. However, genetic screening libraries keep growing beyond genome scale²¹ and often contain tens to hundreds of millions

of cells, a scope most of these techniques are currently incompatible with. A widespread strategy to overcome this limitation is to only isolate cells with an interesting phenotype from the library for subsequent genotyping.²² In the past, this paradigm has largely limited cell-based genome-wide screens to three types of easily selectable phenotypes: proliferation rate, cell death inhibition, or a change in fluorescence intensity compatible with fluorescence-activated cell sorting (FACS).^{23–26}

Increasingly powerful microscopic imaging provides information-rich data on diverse cellular phenotypes^{27–29} and would be an ideal technology to read out biological phenotypes of interest,^{30,31} particularly if combined with recent advances in machine learning. However, its application in genome-wide forward genetic screening has been hampered by a lack of scalability and other limitations. “*In situ* sequencing by synthesis” (ISS) sequences short perturbation-encoding DNA barcodes directly in cells.^{32,33} Because ISS does not include an enrichment step for

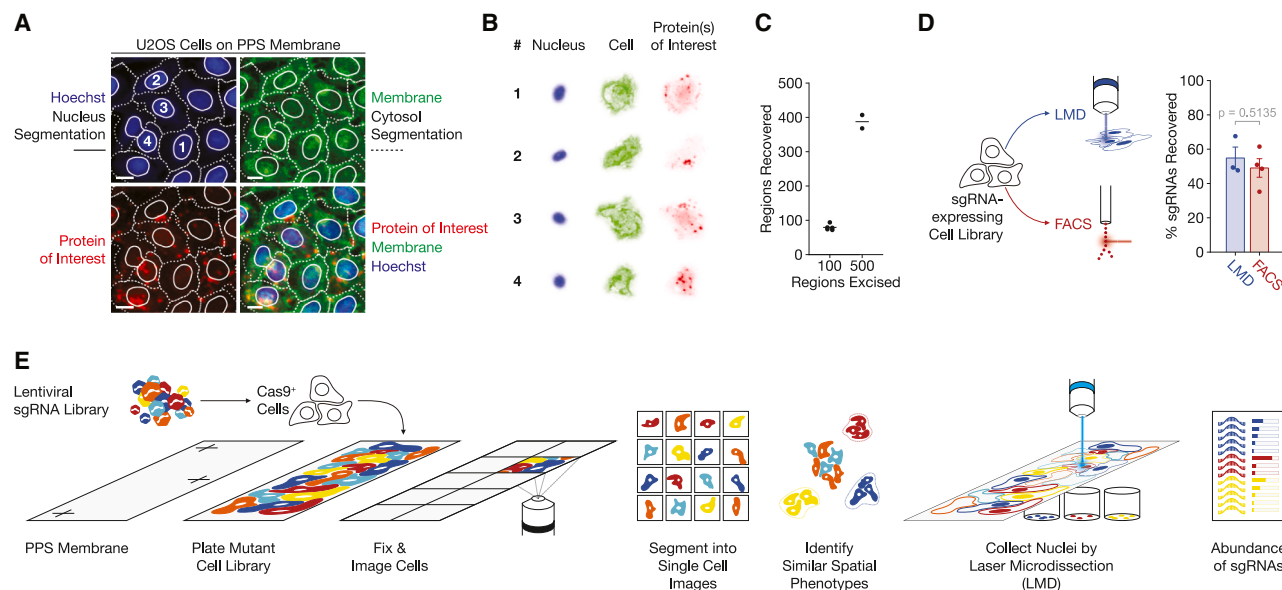


Figure 1. SPARCS enables scalable CRISPR screening for spatial phenotypes in human cells

(A) Example images of U2OS cells on PPS membranes. Solid lines indicate nuclear segmentation based on Hoechst DNA staining; dotted lines indicate cytosol segmentation based on WGA-Alexa 488 staining (plasma membrane). Numbers correspond to images of individual cells shown in (B). Images were acquired on an Opera Phenix microscope in confocal mode with 20 $\times$ magnification. Scale bars represent 15 μ m. PPS: polyphenylene sulfide.

(B) Post-segmentation images of individual mCherry-LC3-expressing U2OS cells. Numbers correspond to cells shown in (A).

(C) 100 or 500 regions were excised from U2OS cells grown on a PPS membrane slide and subsequently counted. Five and two technical replicates were excised from one slide, respectively.

(D) Comparison of sgRNA recovery after isolation of sgRNA-expressing fixed cells from one library either by LMD (Leica LMD7, 1,000 nuclei per replicate, 3 independent biological replicates) or FACS (technical replicates). Bars indicate mean % sgRNAs recovered; error bars indicate SEM. p value was calculated with an unpaired two-tailed t test.

(E) Overview of genome-wide CRISPR screening for microscopy-based phenotypes with the SPARCS pipeline. Instructions for LMD of selected cells are generated using our open-source Python library py-lmd. PPS: polyphenylene sulfide.

selected phenotypes, all cells in a mutant library must be sequenced regardless of whether they display a phenotype, limiting its ability to screen large library sizes at high coverage.^{34–42} Image-based flow cytometers with sorting capabilities have recently enabled the investigation of spatial phenotypes at high throughput.^{43–45} These devices rely on low-resolution flow-based microscopy of detached cells, preventing the identification of complex phenotypes. In addition, this technology makes sorting decisions in real time, restricting it to the identification of predefined phenotypes and preventing reanalysis of past screens. Another method photoactivates fluorophores to mark cells for subsequent isolation by FACS.^{46–50} This approach can only separate a few different phenotypes by fluorophore brightness⁵¹ and requires real-time decisions on which cells to isolate, preventing whole-dataset analysis to discover unexpected new phenotypes.

To enable scalable genetic screening for image-based phenotypes, we set out to develop a technology that meets four key requirements: first, it should work on cells *in situ* with state-of-the-art microscopy techniques. Second, it should accommodate large screening libraries to ensure adequate representation of rare phenotypes. Third, it should be compatible with the unbiased identification of previously unknown phenotypes from entire image datasets rather than single images in real time.

Fourth, it should allow for reanalysis and reselection of cells from previous archived screens to allow the application of novel image analysis methods to previously performed screens as they become available.

RESULTS

Scalable spatial genotyping by laser microdissection

To analyze the spatial composition of tissues and clinical samples by mass spectrometry, we have been advancing workflows based on laser microdissection (LMD), a technique that uses a focused UV laser to cut out and collect arbitrary shapes from tissue sections.^{52,53} In a recent development, deep visual proteomics (DVP), we use LMD to excise defined tissue regions for subsequent proteomic characterization of individual cell types or extracellular zones by mass spectrometry.^{54–56} We reasoned that the isolation of single phenotypically interesting cells from a pooled library by LMD would provide an ideal basis for a forward genetic screening technology for spatial phenotypes.

LMD requires samples to be on a membrane that can be cut by a UV laser, so we first tested whether cells could be grown and imaged directly on such polymer membranes. Indeed, on polyphenylene sulfide (PPS) membranes, spinning disk confocal microscopy produced high-quality images with little

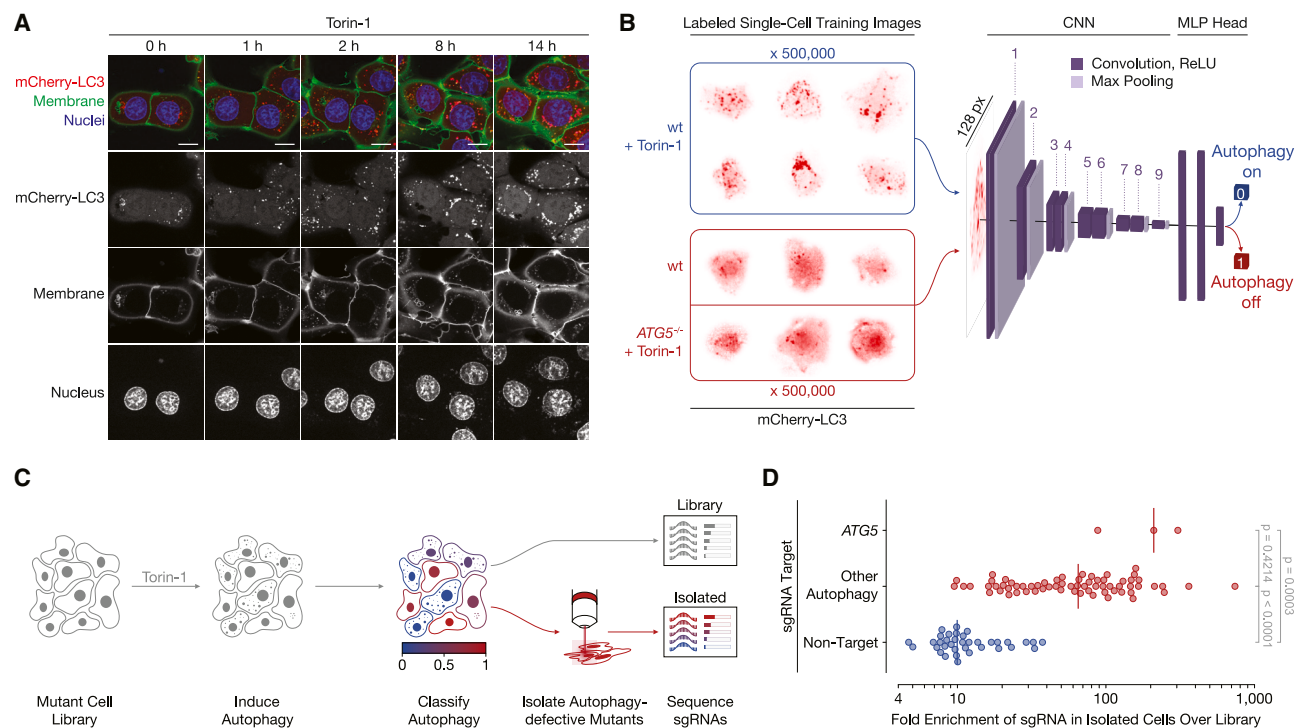


Figure 2. SPARCS achieves strong enrichment of spatial phenotypes in a forward genetic screen

(A) U2OS cells expressing mCherry-LC3 and mNeon tagged with the lipidation signal of Lck at the N terminus (membrane marker) were stimulated with Torin-1 and imaged live once per hour on a Nikon Eclipse Ti2 confocal microscope with 100 $\times$ magnification. Scale bars represent 15 μ m. One representative of three independent experiments.

(B) Overview of classifier 1.0 for autophagic distribution of mCherry-LC3 in single U2OS cells. CNN: convolutional neural network; MLP: multilayer perceptron.

(C) Overview of SPARCS screening for autophagy.

(D) Results from a SPARCS screen for autophagosome formation on 1.2 million U2OS cells. Vertical lines represent medians of the respective groups. *p* values were calculated with a Kruskal-Wallis test followed by Dunn's test.

background fluorescence that showed normal cellular morphology (Figures 1A and S1A). By segmenting these images into individual cells, we generated multi-channel perturbation image datasets from which we aimed to identify cells with phenotypes of interest for genotyping (Figure 1B). We then developed a rapid cutting protocol for LMD that is compatible with subsequent genotyping, reaching a speed of 1,000 nuclei per hour.

Counting excised membrane regions collected in a microwell plate using this protocol showed a yield of approximately 80% (Figure 1C). To test the genotyping of excised nuclei, we generated a pool of U2OS cells, each tagged with one of 77,441 unique sgRNAs in the Brunello CRISPR library integrated into the genome.⁵⁷ After imaging, stitching into whole-slide images (WSIs), and segmentation of nuclei, we performed automated LMD excision of 1,000 individual nuclei from this library with our newly developed open-source Python library py-lmd (Figures S1B and S1C). Lysis and PCR amplification of the integrated sgRNA followed by sequencing identified 549 unique sgRNAs on average in these lysates, demonstrating that isolating individual nuclei for subsequent CRISPR genotyping is feasible with LMD (Figure 1D). Comparing the number of unique sgRNAs in the post-LMD lysate with a lysate of the same number of fixed cells from the same library isolated by FACS showed that both techniques recovered an sgRNA from

approximately 50% of cells (Figure 1D). From these data, we concluded that potential DNA damage induced by LMD does not hamper sgRNA recovery. To determine the accuracy of nuclei excision with our rapid LMD protocol in different cell lines, we fluorescently labeled and then mixed populations of cells expressing different sgRNAs. Excising the nuclei of one population and then sequencing their sgRNAs showed at least 95% of sequencing reads to map to the excised population (Figures S1D and S1E). In summary, our results demonstrate that it is possible to employ LMD to recover genetic information from imaged cells at a throughput compatible with genetic screening (Figures 1E and S2). We call this approach spatially resolved CRISPR screening (SPARCS).

Validating SPARCS for genetic screening

To further develop SPARCS, we applied it to screen for regulators of starvation-induced macroautophagy (hereafter referred to as autophagy), a fundamental process for cellular energy management.^{58,59} The signature of autophagy is the formation of vesicles called autophagosomes that are covalently decorated with proteins from the ATG8 family, including the well-studied human protein LC3B. A critical component of this conjugation is the E3-like ATG12-ATG5 complex that mediates the covalent attachment of LC3B to phosphatidylethanolamine. To

follow the formation of autophagosomes during starvation, we stably expressed LC3B tagged with mCherry (mCherry-LC3) in U2OS cells. When treated with the mTOR inhibitor Torin-1 to mimic starvation, which induces autophagy, these cells accumulated mCherry-LC3-positive puncta (Figure 2A).

In a genetic screen, cells expressing sgRNAs against essential regulators of autophagy are unable to form these puncta. To identify these cells, we trained a deep learning-based image classifier to differentiate between cells with or without autophagosomes (Figure 2B). The training dataset was composed of single-cell images of mCherry-LC3-expressing U2OS cells treated with Torin-1 (autophagy-on class) or left untreated (autophagy-off class). As an additional group, we introduced cells treated with Torin-1, yet deficient in ATG5 (autophagy-off class). We used images from several biological replicates to avoid overfitting of our classifier to batch-specific characteristics such as staining intensity or cell density (Table S1). To evaluate the performance of this classifier 1.0, we generated a new test dataset of images from unstimulated and Torin-1 stimulated wild-type and *ATG5*^{-/-} mCherry-LC3-expressing U2OS cells that had not been part of the training set. Classifier 1.0 achieved a false discovery rate (FDR) of <1% (Figure S3A), meaning that less than 1% of cells classified as potential hits with an autophagy-off phenotype were false positives that came from the autophagy-on class.

We then validated SPARCS by performing a small pilot screen on autophagosome formation in 1.2 million Torin-1-stimulated mCherry-LC3 U2OS cells transduced with the Brunello CRISPR knockout (KO) library (Figure 2C). From this library, we isolated the top 0.1% of cells classified as autophagy-off by classifier 1.0 with a score >0.94, corresponding to a test set FDR of 0.38%. Compared with the entire library, we found sgRNAs targeting *ATG5* to be highly enriched among isolated cells (median 200-fold) (Figure 2D). sgRNAs targeting other autophagy-related genes had a median of 60-fold enrichment, with the most strongly enriched sgRNAs exceeding a 700-fold enrichment (Figure 2D). Control sgRNAs not targeting any human genes (“non-targeting controls” [NTCs]) were rare among isolated cells with a median enrichment of 10-fold (Figure 2D). To analyze the reproducibility of SPARCS, we screened a focused library targeting the hits of this pilot screen in two replicates. We found the recovery of hit sgRNAs to be consistent between replicates ($R^2 = 0.99$), showing that screening with SPARCS yields reproducible results (Figure S3B). Together, these results confirm that the SPARCS protocol stitches and registers WSIs with sufficient accuracy for the isolation of specific nuclei of interest. They also demonstrate that assessing autophagosome formation based on images is feasible with a deep learning classifier and that in SPARCS, this classifier can be used to screen for autophagosome formation.

Accurate detection of autophagy defects in single-cell images

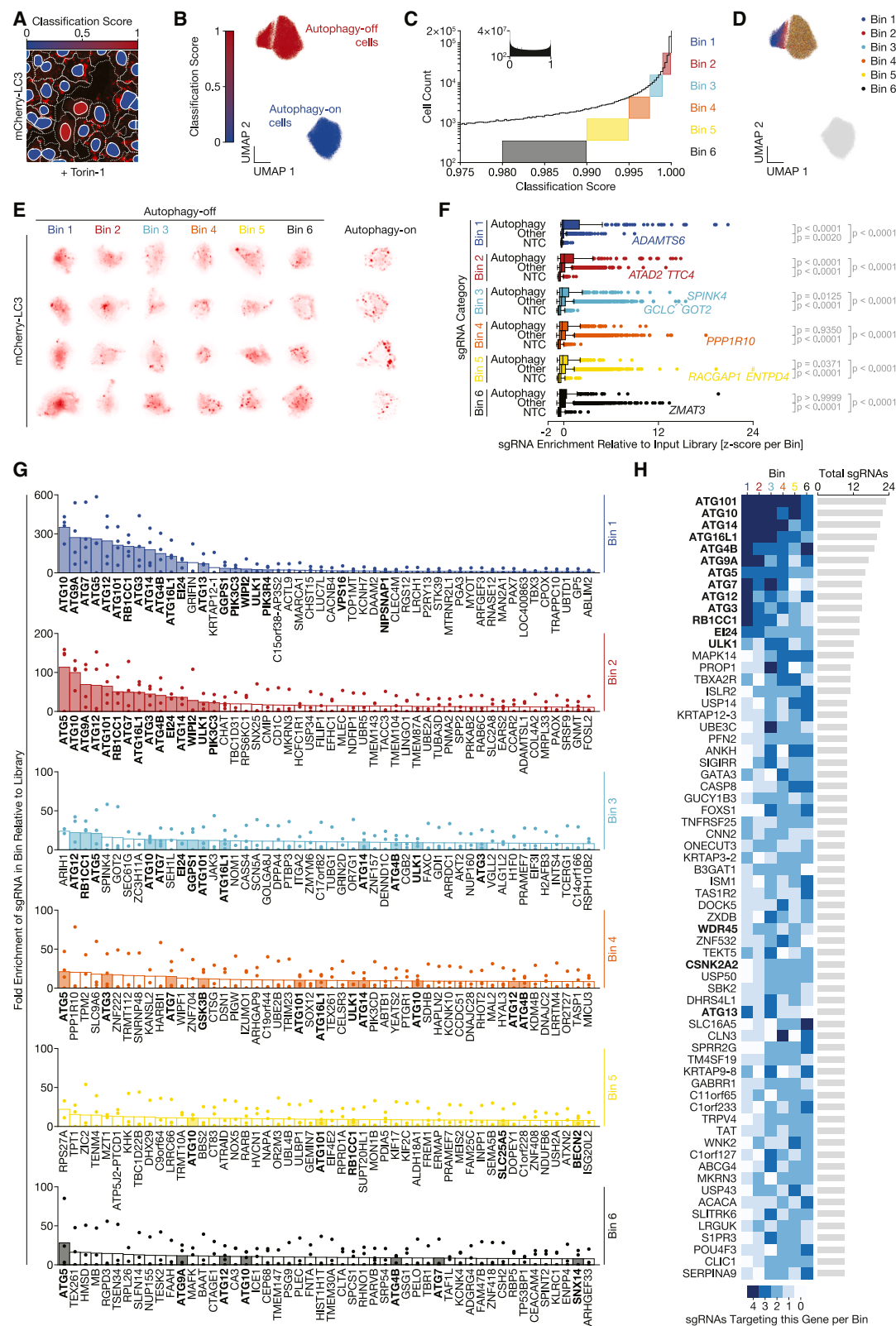
A classifier’s receiver operating characteristic (ROC) curve visualizes the trade-off between true positive rate (the fraction of all autophagy-off cells that are correctly identified) and false positive rate (the fraction of autophagy-on cells incorrectly predicted as autophagy-off). The ROC curve of our classifier 1.0

confirmed its overall accuracy with an area under the curve (AUC) of >0.92 (Figure S3C). However, at 1% FDR (= 99% precision), the recall (= true positive rate) of classifier 1.0 was below 26% (Figure S3C). Closer analysis of the different categories of cells in the test dataset revealed that the classifier excelled at identifying autophagy-on cells but performed poorly at recognizing autophagy-off cells (Figures S3D and S3E).

To improve classification of autophagy-off cells, we refined our staining and imaging protocol and pre-filtered the unstimulated and Torin-1-stimulated images for autophagy-off and -on cells to minimize the number of mislabeled training examples (Figures S4A and S4B). We then trained a new version 2.0 of our classifier (Figure S4B; Table S1). Evaluating this classifier, we found that Torin-1-stimulated wild-type cells, unstimulated wild-type cells, and *ATG5*^{-/-} cells clustered separately in its internal representations, most prominently in the 8th of 9 convolutional layers (Figure S4C). These results suggested that our convolutional neural network (CNN) had now learned to featurize images of LC3 distribution in a way that enables accurate classification of cells undergoing autophagy. Of note, classifier 2.0 could discriminate between *ATG5*^{-/-} and unstimulated wild-type cells despite those cells being in the same training class (Figure S4C), a clear improvement over classifier 1.0 (Figure S3F). Its ROC curve was also drastically improved with an AUC of >0.999 (Figure S4D). Remarkably, in the binary classification output, almost all cells were correctly classified according to their autophagy status even with a simple classification score threshold of 0.5 (Figures S4E and S4F). With classifier 2.0, classification thresholds >0.98 produced FDRs of <1%, with higher thresholds reducing the FDR further without yet diminishing the excellent recall of nearly 100% (Figures S4G and S4H). Thus, for a complex biological process such as autophagy, training a CNN-based classifier on images from comparatively few biological replicates achieves excellent performance. To demonstrate the wide applicability of SPARCS, we trained classifiers with the same architecture to distinguish morphologies of the Golgi apparatus based on images of HeLa cells on PPS membranes stained with antibodies against the cis-Golgi marker protein GM130. To model different Golgi morphologies, we treated the cells with the microtubule polymerization inhibitor nocodazole or the Arf-guanine exchange factor inhibitor brefeldin A (BFA) (Figures S5A and S5B). Similar to the autophagy classifier, these classifiers achieved AUCs of 0.988 (nocodazole) and 0.999 (BFA), respectively (Figures S5C and S5D).

Genome-wide autophagy screen with SPARCS

Encouraged by these results, we used SPARCS to conduct a genome-wide screen on autophagosome formation and distribution. We screened a library of 40 million mCherry-LC3-expressing U2OS cells at a median coverage of 1,818 cells per gene in the human genome in batches of 5 and 35 million cells (Figure S6). Classifying autophagy based on the distribution of LC3 within the first batch showed that 0.56% of cells had a score >0.98. We regarded these cells as potential autophagy-defective hits and, upon examining the 8th CNN layer featurization of their LC3 distribution using parametric uniform manifold approximation and projection (UMAP),⁶⁰ found them to cluster separately from autophagy-on cells in the library with a classification score



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<0.02 (Figures S7A and S7B). For genotyping, we divided the hits into six bins according to their classification score (Figure S7C): the top bin represented a cutoff at which we found *ATG5*^{−/−} to be strongly enriched in our test dataset, whereas the second bin corresponded to unstimulated wild-type cells. Bins 3–6 contained the remaining potential hits with a roughly equal number of cells per bin. Analyzing the 8th CNN layer featurization of the LC3 distribution in the potential hits revealed that cells in bins 1 and 2 clustered separately from bins 3–6 (Figure S7D). This indicated that they contained different LC3 distribution phenotypes, potentially corresponding to stronger defects in autophagosome formation. Indeed, we observed the fewest LC3 puncta in cells from bins 1 and 2 (Figure S7E).

For the second genome-wide screen batch, we refined our classifier by more stringently selecting training examples of autophagy-on and -off cells, thereby further improving its overall performance (Figures S8A–S8D). Using this new classifier 2.1, we obtained similar results from the second batch compared with classifier 2.0 on the first screen batch: 1.40% of cells were classified as autophagy-off with a score above 0.98, and in their 8th CNN layer featurization, these cells again clustered separately from their autophagy-on counterparts with a score <0.02 (Figures 3A and 3B). We therefore applied the same binning strategy to these images (Figures 3C–3E) and then isolated a total of 395,173 nuclei across both screen batches to sequence their sgRNAs. Given their similarity on the phenotypic level, we analyzed the genetic data of both batches together. All bins showed a marked enrichment of targeting over non-targeting sgRNAs and enrichment scores up to 600-fold, promising the identification of autophagy-relevant genes (Figures 3F and 3G; Table S2). In line with our FDR calculation (Figures S4G and S8B) and our conclusions from the featurization of individual images (Figures 3D and S7D), sgRNAs targeting genes known to be involved in autophagosome formation were most strongly enriched in bins 1 and 2 (Figure 3F). *ATG5*, whose KO our classifier was trained to identify, was among the most highly enriched genes in several bins, validating our supervised classification approach for a genome-scale screen (Figure 3G). The Brunello library targets each

gene in the human genome with four sgRNAs. While in the higher score bins 1 and 2, genes with a high mean enrichment score had several sgRNAs enriched; in lower bins, genes with a relatively high mean enrichment score often only had a single highly enriched sgRNA, indicating potential off-target effects. This prompted us to evaluate the number of sgRNAs enriched per gene, treating sgRNAs as replicates and then ranking hit genes by their respective enrichments and *p* values as alternative metrics to score screening hits. Here, we again found the strongest hits to contain many autophagy-related genes (Figures 3H and S8E). Taken together, these results establish that SPARCS is highly effective for large-scale genetic screens on spatial phenotypes. Furthermore, despite the inherent complexity of image-based phenotypes, our supervised classifier facilitated the enrichment of a very small subset of individual cells with a genetically defined phenotype from a diverse genome-wide library of 40 million cells.

EI24 reorganizes membranes for autophagy

Remarkably, our screen recovered almost all core genes of the starvation-induced macroautophagy pathway. This included the complete ULK1 complex and LC3 lipidation cascade (Figure 4A). Closer inspection of individual hits revealed that the most strongly enriched gene that is not a canonical macroautophagy gene was *EI24*, a gene coding for an endoplasmic reticulum (ER)-resident transmembrane protein⁶¹ (Figure 4B). *EI24*^{−/−} cells have previously been described as autophagy-defective, but with a phenotype resulting in spontaneous LC3 puncta formation.⁶² This finding is not in line with the 82-fold enrichment of *EI24* in our screen, given that our classifier was trained to recognize cells with impaired rather than increased autophagosome formation. To investigate why we found *EI24* KOs enriched among cells classified as autophagy-off, we generated individual *EI24*^{−/−} clones. Consistent with the previously reported spontaneous LC3 puncta formation, *EI24*-deficient cells have been described to exhibit increased lipidation of LC3 under steady-state conditions,⁶² a phenotype we confirmed in *EI24*^{−/−} clones (Figure 4C). However, in contrast to previous reports, we found LC3 puncta formation in response to Torin-1 to be largely

Figure 3. Genome-wide CRISPR screening for autophagosome formation in 40 million U2OS cells using SPARCS

- (A) Example region from a genome-wide SPARCS screen on autophagosome formation in mCherry-LC3-expressing U2OS cells after Torin-1 stimulation for 14 h. Colors in nuclei indicate the result of autophagy classification; dotted lines indicate cytosol segmentation. Images were acquired on an Opera Phenix microscope in confocal mode with 20× magnification.
- (B) UMAP representation of single-cell images from all cells in screen batch 2 with a classification score ≥ 0.98 (red) or <0.02 (blue) featurized through the first 8 convolutional layers of autophagy classifier 2.1. 91,320 images are depicted for each category.
- (C) Histogram of autophagy classification scores in the genome-wide CRISPR KO library batch 2 (inset) zoomed in on cells classified as autophagy-off with a score above 0.975. Colored boxes illustrate the binning strategy we used to isolate cells for sgRNA sequencing.
- (D) UMAP from (B) colored according to our binning strategy along different autophagy classification thresholds. 15,220 images are depicted per bin.
- (E) Images of individual cells from each bin in screen batch 2.
- (F) Z scored enrichments of individual sgRNAs in each bin from batches 1 and 2. Vertical lines depict the median, boxes depict interquartile range (IQR), and whiskers depict 1.5× IQR. #: one sgRNA targeting *ENTPD4* with a Z score of 42.1 in bin 5 is not depicted. *p* values were calculated with a Kruskal-Wallis test followed by Dunn's test. NTC: non-targeting control.
- (G) sgRNA sequencing results of the top 50 genes in each of the six bins filtered for genes for which we found at least two different sgRNAs in the respective bin in batches 1 and 2. Enrichment is calculated as the fraction of reads for an sgRNA in the respective bin divided by the fraction of reads of that sgRNA in the entire library. Bars indicate average enrichment per gene calculated from the enrichment of individual sgRNAs indicated as dots. Filled bars depict autophagy-related genes highlighted in bold.
- (H) Number of different sgRNAs per gene in each bin for all genes with at least 9 total sgRNAs across all bins. sgRNAs were counted if they were sequenced with a read fraction in the top 50% per bin. Bars on the right show the sum of sgRNAs across all bins, counting the same sgRNA multiple times if it appears in multiple bins.

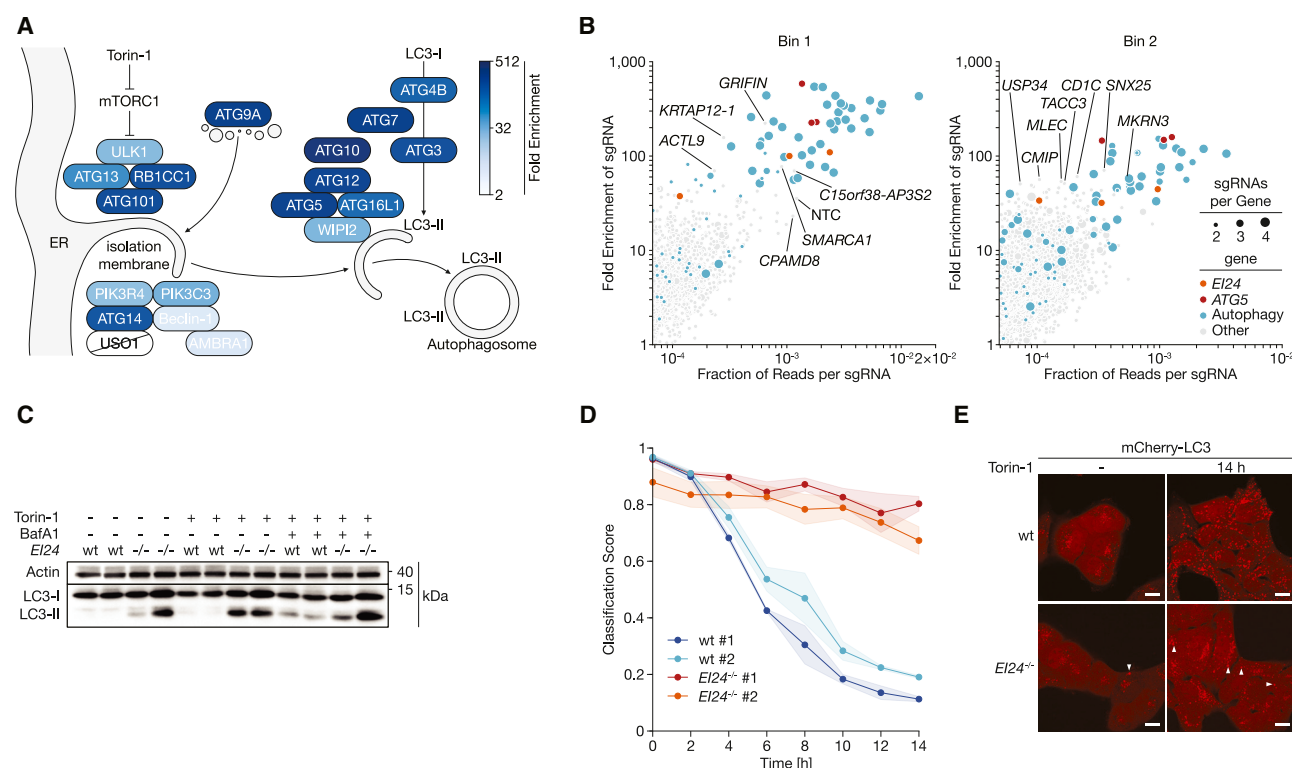


Figure 4. Analysis of hits from genome-wide autophagosome formation SPARCS screen

(A) Overview of the canonical macroautophagy pathway. Colors indicate the highest enrichment value we found for a given gene in any bin. *USO1* was not found with at least two different sgRNAs in any single bin.

(B) Enrichment vs. read fraction for individual sgRNAs in the top two bins for genes where we found at least two different sgRNAs in the respective bin. Both screen batches are depicted together. Circle sizes indicate the total number of different sgRNAs we found for a given gene, colors indicate different groups of genes. Individual sgRNAs from the “other” group are annotated. NTC: non-targeting control.

(C) Immunoblot of endogenous LC3 lipidation in wild-type and *El24*^{-/-} mCherry-LC3 and LckLip-mNeon-expressing U2OS cells stimulated with Torin-1 and bafilomycin A1 for 2 h. Two clones are shown per genotype. One representative of three independent experiments.

(D) Time-course analysis of autophagy classification in clones of wild-type and *El24*^{-/-} mCherry-LC3 and LckLip-mNeon-expressing U2OS cells. Cells were treated with Torin-1 for up to 14 h. Dots represent average classifier 2.1 scores from cells in 15 fields of view per time point and clone from three independent experiments; shaded areas represent SEM. Images were acquired on an Opera Phenix microscope in confocal mode with 20× magnification.

(E) Microscopy images of live mCherry-LC3 and LckLip-mNeon-expressing wild-type and *El24*^{-/-} U2OS cells untreated or after 14 h of Torin-1 stimulation. Arrowheads indicate larger mCherry-LC3 aggregates. Images were acquired on a Nikon Eclipse Ti2 confocal microscope with 100× magnification. Scale bars represent 15 μm. One representative of three independent experiments.

abolished in *El24*-deficient cells (Figures 4D and S8F). Instead, these cells formed a single mCherry-LC3-positive speck that became more pronounced with Torin-1 stimulation, indicating a general defect in membrane traffic or autophagosome formation (Figure 4E). These results explain why our classifier picked up *El24* KO and demonstrate again that even supervised image classification can identify previously undescribed phenotypes. Our results further indicate that *El24* is required for autophagosome formation and, beyond its recently described role in maintaining Ca^{2+} homeostasis across the ER membrane,⁶³ has a function that remains to be investigated.

Genome-wide screening for STING trafficking with SPARCS

Having demonstrated the ability of SPARCS to exhaustively map the genetics of complex cellular phenotypes, we next applied it to identify regulators of the innate immune sensor STING.⁶⁴

Following its activation by cyclic dinucleotides such as cGAMP,^{65–67} STING initiates multiple downstream pathways, including type-I interferon signaling, nuclear factor κ B (NF- κ B)-mediated inflammatory responses, autophagy,⁶⁸ and pyroptotic cell death.⁶⁴ Thereby, STING plays central roles in antimicrobial defense, inflammation, and cellular senescence.^{64,69}

STING signaling is tightly linked to its intracellular trafficking. Under resting conditions, STING resides in the ER membrane. Upon activation, STING exits the ER in COPII vesicles^{70–72} and traffics through the ER-Golgi intermediate compartment (ERGIC), the Golgi, and eventually lysosomes, where STING is degraded.^{73,74} Recent studies have shown that STING activation is accompanied by an increase in Golgi pH that is required for autophagy and cell death induction.^{75–77} These findings suggest that the relationship between STING localization and signaling remains incompletely understood. Because SPARCS directly captures subcellular localization phenotypes, we reasoned that

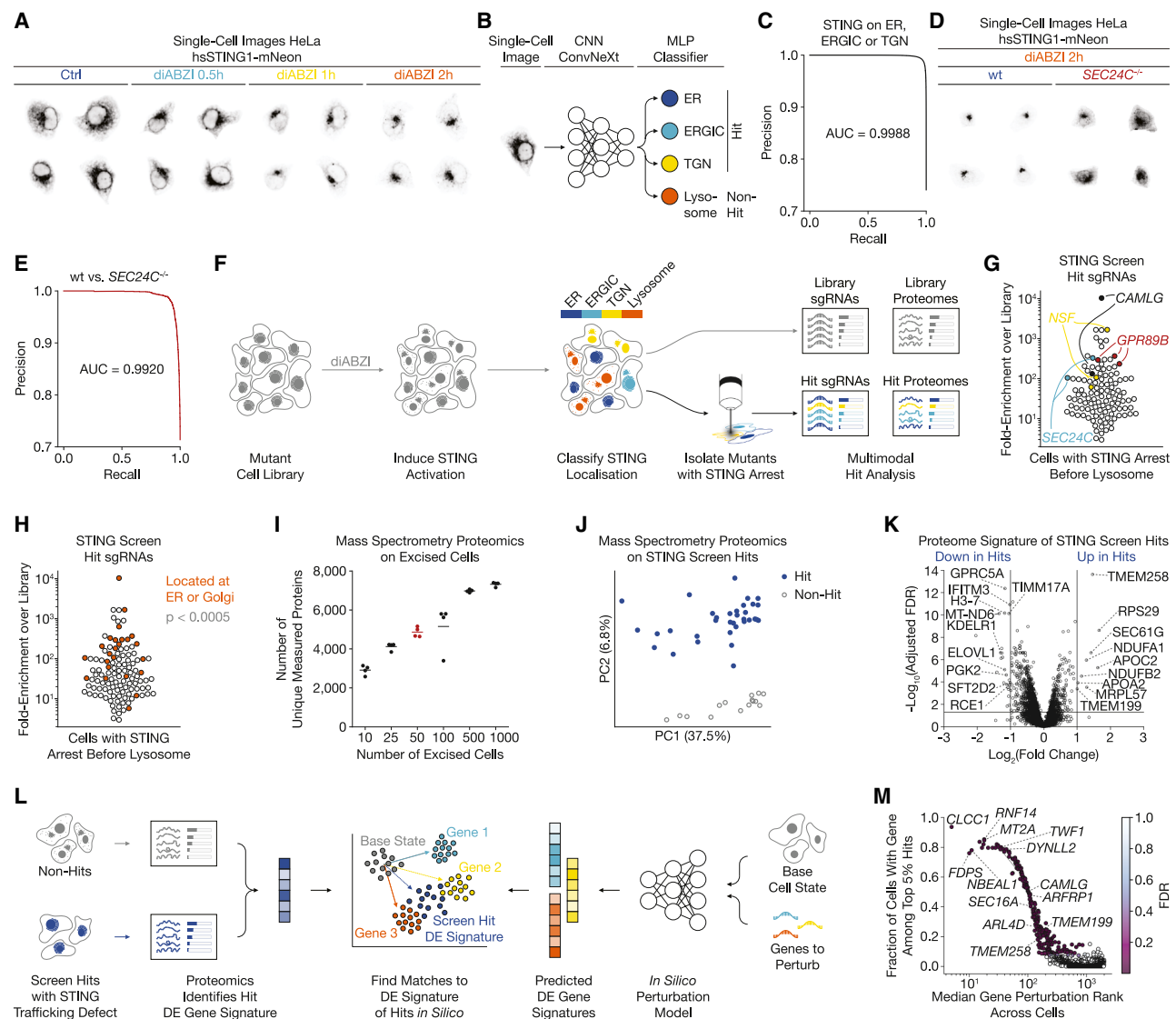


Figure 5. A genome-wide SPARCS screen for STING trafficking integrating proteomics and in silico perturbation prediction

(A) Single-cell images of HeLa cells stably expressing hsSTING1-mNeon treated with diABZI for the indicated times corresponding to STING localization on ER, ERGIC, TGN, or the lysosome.

(B) Schematic of the 4-class subcellular STING localization classification approach for single-cell images as in (A) based on the fine-tuned ImageNet-pretrained model ConvNeXt with a custom classification head. CNN: convolutional neural network; MLP: multilayer perceptron.

(C) Precision-recall curve for identifying the localization of STING on the lysosome vs. ER, ERGIC, or TGN using the classifier in (B).

(D) Single-cell images of *SEC24C*^{-/-} hsSTING1-mNeon HeLa cells stimulated with diABZI for 2 h.

(E) Precision-recall curve for identifying wild-type vs. *SEC24C*^{-/-} hsSTING1-mNeon HeLa cells using the classifier in (B).

(F) Schematic of our genome-wide SPARCS screen for STING trafficking combined with mass spectrometry. From 29.4 million cells we isolated 2,071 predicted hit cells for genotyping and conducted proteomics on 2,128 cells in bins of 40–60 morphologically similar cells.

(G) sgRNAs enriched in the STING screen from (F).

(H) sgRNAs enriched in the STING screen from (F) that correspond to genes encoding proteins located at the ER or Golgi. The *p* value for enrichment of sgRNAs targeting genes encoding proteins localized to the ER or Golgi was calculated using a one-sided hypergeometric test.

(I) Number of unique detected proteins by LC-MS for different numbers of cells excised by SPARCS. Horizontal lines indicate means of separate experiments depicted by individual dots.

(J) PCA of proteomes of hit- and non-hit (classifier score ≤ 0.01) cells from the STING screen.

(K) Mass spectrometry-based proteomics results from (F) comparing the proteome of all excised hits to STING classifier-predicted non-hits. FDR was Benjamini-Hochberg adjusted.

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it would enable sensitive identification of genetic regulators of STING trafficking beyond past screens.^{75,76,78–82}

To track STING trafficking by microscopy, we expressed a fluorescent hsSTING1-mNeonGreen fusion protein in HeLa cells. Upon stimulation with the synthetic STING agonist diABZI,⁸³ STING redistributed from the ER to small peripheral lysosome-like puncta within 2 h, recapitulating its reported trafficking dynamics (Figure 5A).⁷³

To identify cells with defects in STING trafficking, we trained a single-cell image classifier to assign hsSTING1-mNeon localization to four major compartments based on time-course imaging data: ER, ERGIC, *trans*-Golgi network (TGN), and endolysosomal vesicles. These localization states approximately corresponded to 0, 0.5, 1, and 2 h of diABZI stimulation, respectively (Figure 5B). Evaluation on a held-out test set showed that the classifier most accurately identified the terminal endolysosomal localization state relative to all other compartments (Figures 5C, S9A, and S9B). This likely reflects the comparatively homogeneous STING distribution observed across cells at this final trafficking stage. To assess classifier performance in a genetic perturbation setting, we tested whether it could distinguish HeLa hsSTING1-mNeon cells lacking SEC24C, a known regulator of STING trafficking,^{68,84} from wild-type cells (Figure 5D). The classifier robustly separated SEC24C^{−/−} and wild-type cells (AUPRC = 0.993; precision at 90% recall = 99.34%), corresponding to an estimated hit identification sensitivity of 90% recall at an FDR of 1.3% in a genome-wide screen (Figure 5E).

We then conducted a genome-wide SPARCS screen for STING trafficking, using this classifier to identify screen hits, i.e., cells that show STING localization on the ER, ERGIC, or TGN instead of the endolysosome after 2 h of diABZI treatment (Figure 5F). Screening a library of 29.4 million HeLa hsSTING1-mNeon cells and excising 2,071 hit mutants, our classifier enriched KO of known STING trafficking factors such as SEC31A⁸⁵ and the positive control SEC24C several hundredfold (Figures 5G and S9C; Table S3). Additional genes were highly enriched for which no mechanism of STING regulation is known, such as the SNARE recycling factor NSF (SEC18), the TRAPP-III complex member TRAPPC11, and the Golgi-resident Cl[−] channel GPR89B. Overall, we found genes encoding proteins located on the ER or the Golgi strongly enriched among hits (Figure 5H).

Profiling of SPARCS screening hits by mass spectrometry-based proteomics

We reasoned that the physical isolation of cells by SPARCS could be leveraged to phenotypically characterize screening hits, enabling interrogation of cellular behaviors and molecular states beyond the spatial resolution provided by imaging alone. Since LMD-excised cells retain intact proteins,⁸⁶ we used high-

sensitivity liquid chromatography-mass spectrometry (LC-MS)-based proteomics to profile protein abundance in STING screen hit cells (Figure 5F). We reproducibly quantified approximately 5,000 proteins from 50 cells (Figure 5I). These results establish that SPARCS-isolated material is compatible with sensitive downstream proteomic analysis even at very low cell numbers. We next analyzed 43 replicate pools, each containing 40–60 morphologically defined, classifier-predicted hit cells, and compared their proteomes to non-hit cells. Principal-component analysis (PCA) revealed robust and reproducible separation between the two groups (Figure 5J). Quantitative comparison of hit and non-hit proteomes across our morphological groups identified multiple significantly enriched and depleted proteins (Figure 5K). Consistent with the sgRNAs recovered from hit cells in this screen, these prominently included proteins linked to ER and Golgi function such as SEC61G, TMEM258, and KDELR1, as well as proteins encoded by the screen's strongest hit genes (Figure S9D). Because proteomes were measured only 2 h after diABZI stimulation, these differences are unlikely to reflect transcriptional responses induced by STING signaling. Instead, they likely reflect steady-state proteomic differences caused by the underlying genetic perturbations that alter STING trafficking.

We next asked whether proteomic signatures obtained from SPARCS-isolated cells could be combined with perturbation prediction models to nominate additional candidate regulators of STING trafficking. To this end, we used the *in silico* perturbation model STATE⁸⁷ to predict perturbations with effects mimicking the proteome signature of our STING screen hits, as described in Supplementary Note 1. This proof-of-concept analysis revealed additional ER- and trafficking-associated candidate regulators, including SEC16A, TMEM199 (VMA12), TMEM258, ARFRP1, and CLCC1 (Figures 5L, 5M, and S10A–S10E). Together, these findings demonstrate that SPARCS enables genome-scale identification, isolation, and molecular characterization of cells with complex subcellular phenotypes.

Golgi pH-dependent regulation of STING trafficking by GPR89A and GPR89B

One STING screen hit, a gene called GPR89B, caught our attention. GPR89B and its 99.5%-identical paralog GPR89A encode GPHR, a Golgi-resident Cl[−] channel that helps maintain the acidic luminal pH of the Golgi by mediating counterion conductance.⁸⁸ GPHR deficiency has been shown to cause Golgi de-acidification,⁸⁸ and de-acidification of the Golgi by activated STING has recently been described as necessary for the induction of lysosomal cell death and autophagy by STING.^{75–77} Imaging the intracellular localization of STING, we found strong retention on the ER in GPR89A^{−/−}; GPR89B^{−/−} (hereafter called GPR89 dKO) HeLa cells after stimulation with diABZI (Figure 6A). Stimulation with the STING agonist Compound 53 (C53) leads

(L) Schematic for *in silico* screening to identify genetic perturbations causing the proteomics signature from (K). After multiplying the STING screen hit protein fold changes measured by mass spectrometry with single-cell gene expression data from HeLa cells, predicted perturbation and measured proteomics effects are co-embedded into the prediction space of an *in silico* perturbation model.

(M) Hits from an *in silico* screen of 2,023 gene perturbations aggregated across 300 individual HeLa cells. Colors indicate Benjamini-Hochberg-corrected FDR based on empirical *p* values calculated from rank permutations. *p* values were calculated for both median gene rank and fraction of cells where the gene ranks in the top 5%, and the higher of the two values was used.

Microscopy images were acquired on an Opera Phenix microscope in confocal mode with 40× magnification.

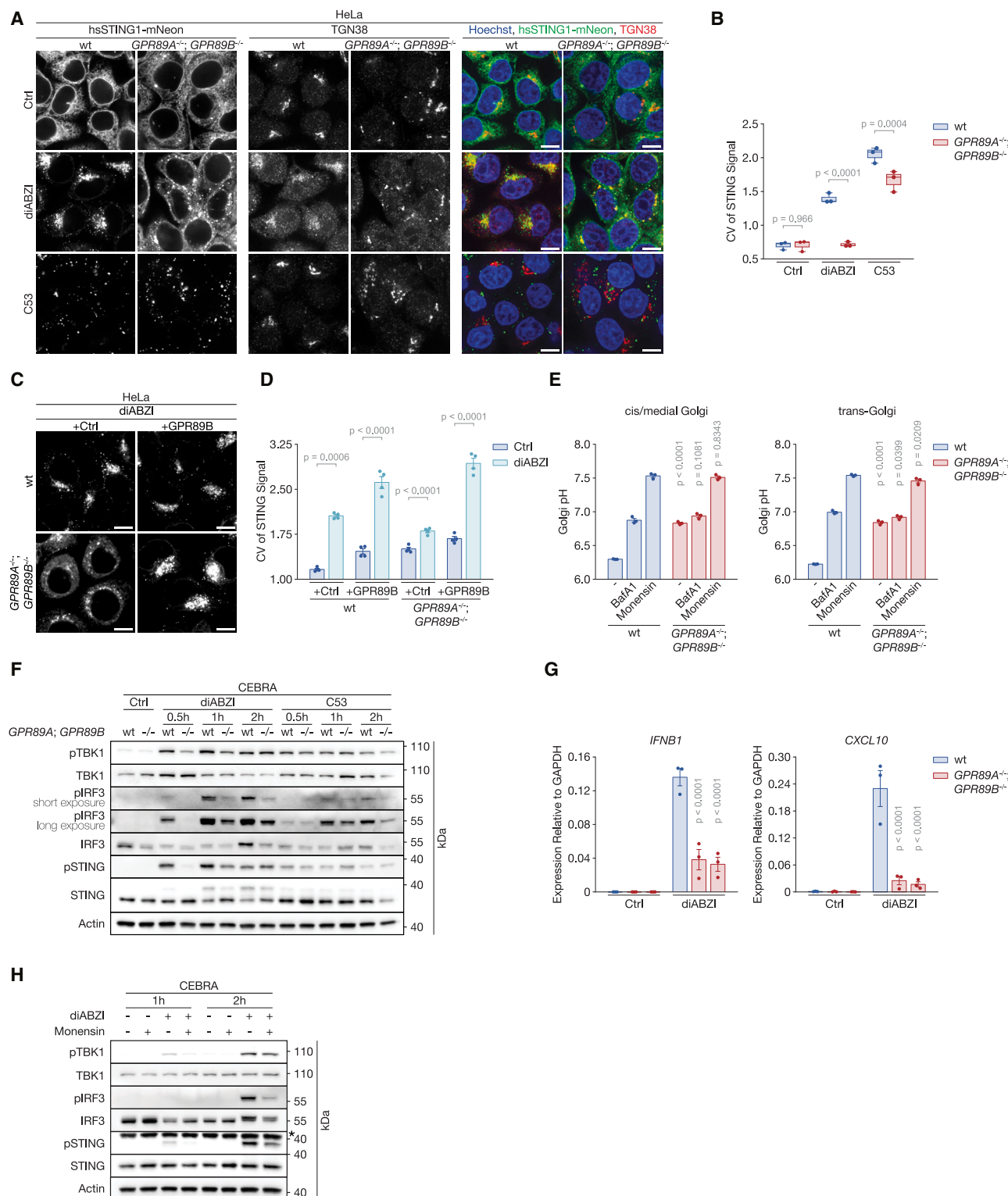


Figure 6. Maintenance of Golgi pH by *GPR89A* and *GPR89B* is required for STING trafficking and activation

(A) Images of *GPR89A*^{-/-}; *GPR89B*^{-/-} HeLa cells stably expressing hsSTING1-mNeon stained for the TGN marker TGN38 stimulated with diABZI or C53 for 2 h. (B) Standard deviation divided by mean (CV) of hsSTING1-mNeon signal from (A). Dots depict three independent experiments. Boxes depict interquartile ranges (IQRs), central lines depict medians, and whiskers extend to extreme values within 1.5 × IQR. (C) Images of *GPR89A*^{-/-}; *GPR89B*^{-/-} HeLa cells stably expressing *GPR89B* stimulated with diABZI for 2 h.

(legend continued on next page)

to STING clustering directly on the ER^{89–91} and was largely unaffected in *GPR89* dKO HeLa cells (Figures 6A and 6B), showing that GPHR specifically regulates trafficking-dependent STING activation.

Expression of *GPR89B* reversed the phenotype of *GPR89* dKO HeLa cells and enabled STING to translocate to the TGN upon diABZI stimulation (Figures 6C and 6D). Measuring Golgi pH in *GPR89* dKO HeLa cells using ratiometric fluorescent probes⁷⁵ showed an increased pH in both the cis/medial Golgi and the TGN, confirming previous results (Figures 6E, S11A, and S11B).⁸⁸ In line with this finding, our *in silico* screen enriched *TMEM199* (*VMA12*), a homolog of yeast V-ATPase assembly factor Vph2p^{92,93} that is localized at the ERGIC.⁹⁴ Mutations in *TMEM199* were recently linked to congenital disorders of glycosylation,⁹⁴ indicating a role in Golgi homeostasis.

To investigate the role of GPHR in antiviral and pro-inflammatory functions of STING, we generated *GPR89* dKOs in CEBRA cells, an *in vitro* monocyte transdifferentiation system we developed similar to BLaER1 cells.^{95,96} Analyzing the phosphorylation of STING and downstream signaling molecules TBK1 and IRF3 via western blot showed a block in activation upon diABZI stimulation in *GPR89* dKO CEBRA cells at early time points (0.5 h) (Figure 6F). Stimulation with C53 led to unimpaired phosphorylation of STING, TBK1, and IRF3, indicating that trafficking-dependent stimuli such as diABZI cannot activate STING when Golgi pH is increased (Figure 6F). In addition to phosphorylation of signaling adaptors, STING-dependent transcription of antiviral (*IFNB1*) and pro-inflammatory (*CXCL10*) signaling molecules was impaired in *GPR89* dKO CEBRA cells (Figure 6G). To exclude that a compensatory mechanism activated by GPHR deficiency rather than increased Golgi pH causes the delay in STING activation, we treated CEBRA cells with monensin, an H⁺ ionophore that de-acidifies the Golgi (Figure 6H). Similar to GPHR deficiency, simultaneous monensin treatment caused delayed phosphorylation of STING, TBK1, and IRF3 in response to diABZI, demonstrating that acute de-acidification of the Golgi can prevent STING activation. Together, these data establish Golgi pH as a new regulatory mechanism of STING trafficking and activation.

DISCUSSION

We present SPARCS, a platform that enables unbiased exploration of the genetic basis of subcellular spatial features in forward genetic screens. At the core of the SPARCS methodology, we have adapted and refined LMD technology to unprecedented throughput to facilitate genetic screening applications. We

have improved the precision and efficiency of isolating single cells from cell cultures while automating the extraction of several hundred thousand nuclei into distinct bins. By integrating AI-based computer vision models, we searched 210 million single-cell images for complex phenotypes. Showing the potential of this approach, the genome-wide SPARCS screens presented here successfully identified nearly all known genes related to macroautophagy, revealed a novel autophagy phenotype associated with the *EI24* gene, uncovered pH-dependent regulation of STING signaling via GPHR, and enabled multimodal characterization of screen hits by mass spectrometry-based proteomics coupled with *in silico* perturbation prediction.

SPARCS offers a unique combination of features that make it a powerful forward genetic screening platform. It can be seamlessly integrated with any state-of-the-art microscope for *in situ* cell imaging. The screening library size is not constrained, except by the imaging microscope's throughput. Consequently, microscopy-based genome-wide perturbation screens can now achieve exceptional coverage. By physically isolating cells, SPARCS enables additional deep phenotypic profiling of hit mutants, for example, by mass spectrometry-based proteomics. Besides the method described here, which involves isolating cells based on predefined classes, SPARCS is also compatible with the identification of individual cells exhibiting entirely novel or unexpected phenotypes via anomaly detection applied to the entire image dataset. Furthermore, in our experience, slides remain suitable for reanalysis after long-term storage, although we did not perform a controlled comparison between fresh and stored samples. This facilitates the exploration of new biological insights within existing data. To streamline LMD, we have developed py-lmd, an open-source Python library for LMD that is available on GitHub.

Our autophagy screen uncovered a potential role in macroautophagy for *EI24*. This gene had previously been implicated in autophagy based on a *C. elegans* screen in 2010, but its mechanism of action had remained unclear.⁶² Beyond the original observation that *EI24* deficiency leads to pronounced formation of non-functional autophagosomes even under steady-state conditions, it was recently suggested that spontaneous Ca²⁺ fluxes across the ER membrane initiate autophagy in *EI24*-deficient cells.⁶³ How spontaneous induction of autophagosome formation in *EI24*^{−/−} cells can be reconciled with a defect in autophagy remained unclear. We found *EI24*^{−/−} cells to form fewer autophagosomes than wild-type cells, suggesting that *EI24* plays a—potentially additional—role in autophagosome formation.

(D) Standard deviation divided by mean (CV) of hsSTING1-mNeon signal from (C). Dots depict separate experiments imaged together. Error bars depict SEM. (E) pH measurement of the indicated Golgi compartments in *GPR89A*^{−/−}; *GPR89B*^{−/−} HeLa cells with ratiometric fluorescent pH sensors. Cells were imaged directly after treatment. BafA1: bafilomycin A1. Dots depict separate fields of view. Error bars depict SEM.

(F) Immunoblot of transdifferentiated *GPR89A*^{−/−}; *GPR89B*^{−/−} CEBRA monocyte lysates following stimulation with diABZI and C53 for the indicated times. One representative of two independent experiments in two clones each.

(G) mRNA quantification of *IFNB1* and *CXCL10* in transdifferentiated *GPR89A*^{−/−}; *GPR89B*^{−/−} CEBRA monocytes following stimulation with diABZI (2 h) via qRT-PCR. Dots depict independent experiments. Error bars depict SEM.

(H) Immunoblot of transdifferentiated CEBRA monocytes following co-stimulation with diABZI and monensin for the indicated times. * denotes a non-pSTING band. One representative of three independent experiments.

Microscopy images were acquired on a Nikon Eclipse Ti2 confocal microscope with 100× magnification. Scale bars represent 10 μm. *p* values were calculated by two-way ANOVA followed by Tukey's test.

Using SPARCS to screen for genes regulating the immune sensor STING, we identified the paralogs *GPR89A* and *GPR89B* as required for STING trafficking from the ER to the Golgi. *GPR89A* and *GPR89B* encode GPHR, a Cl^- channel in the Golgi membrane that acts as a counterion conductor enabling the acidification of the Golgi lumen by V-ATPase.⁸⁸ We found GPHR deficiency to disrupt trafficking-mediated STING activation by diABZI. However, STING activation with Compound 53,⁸⁹ which activates STING directly on the ER without trafficking to the Golgi,⁹¹ remains intact in GPHR-deficient cells. Raising Golgi pH by treatment with the H^+ ionophore monensin phenocopied GPHR deficiency and impaired STING activation with diABZI, establishing a regulatory role for Golgi pH in early STING signaling. Interestingly, an increase in Golgi pH following STING activation has recently been shown to be essential for STING-induced autophagy, lysosomal cell death,^{75,77,79} and protein secretion.⁷⁶ Together with our findings, this suggests that a STING-induced pH increase at the Golgi helps sustain STING activation. Mechanistically, this could be achieved by preventing retrograde trafficking, a known basal inhibitory mechanism that prevents aberrant STING activation and is defective in the autoinflammatory disease COPA syndrome.^{71,72,97}

By physically isolating phenotypically defined cells, SPARCS enables molecular characterization of genetic screen hits beyond the spatial resolution of imaging alone. We leveraged this capability to profile STING screen hits using high-sensitivity MS-based proteomics in parallel with sgRNA sequencing, integrating a genome-wide image-based CRISPR screen with proteomic characterization. This multimodal analysis revealed a shared ER-Golgi disruption signature among hit cells, prominently involving SEC61G, TMEM258, and KDELR1. To nominate additional regulators that our image classifier may have missed, we combined this proteomic signature with the AI-based perturbation model STATE to conduct an *in silico* genetic screen. Although trained exclusively on single-cell transcriptomics data, applying STATE to a proteomics-derived target signature successfully identified additional ER- and trafficking-associated candidates, including CLCC1, a gene recently implicated in ER lipid homeostasis.^{98–100} These results demonstrate that combining high-content imaging, physical cell isolation, and MS-based proteomics creates a multimodal screening paradigm that can reveal regulatory mechanisms inaccessible to any single modality. As efforts to construct AI-based virtual cell models advance, large-scale multimodal perturbation data for training and validation will be increasingly needed. With its open-source tools, compatibility with standard microscopy, phenotyping, and sequencing workflows, and demonstrated ability to interface with computational perturbation models, SPARCS provides an accessible platform for generating such data at genome-wide scale.

Limitations of the study

Although we have significantly optimized LMD for SPARCS, a limitation of the technology as presented here is the 1,000 nuclei per hour excision rate imposed by the currently available free-form excision LMD microscopes. Although single-cell analysis is conceivable, in the current setup we sequenced pools of at least 500 excised cells to improve sequencing fidelity, precluding an individual image-to-sgRNA assignment. Due to the relative abundance of training data, current cellular perturbation pre-

diction models are trained on single-cell RNA sequencing, a domain shift from the proteomics signature we screened for, which may lead to false negatives in the *in silico* screen.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Veit Hornung (veit.hornung@lmu.de).

Materials availability

All unique/stable reagents generated in this study are available from the [lead contact](#) with a completed materials transfer agreement.

Data and code availability

- SPARCS autophagosome and STING screen single-cell image data have been deposited at BioImage Archive: S-BIAD3897. Mass spectrometry proteomics data have been deposited at PRIDE as PRIDE: PXD082653.
- The code described in this manuscript is available from the following GitHub repositories: the py-lmd Python library provides code to direct excision of defined regions on a Leica LMD7 LMD microscope (<https://github.com/MannLabs/py-lmd>). The SPARCStools Python library provides code to rename TIF image files generated by the PerkinElmer Harmony software and stitch these into WSIs (<https://github.com/MannLabs/SPARCStools>). The SPARCSpy Python library contains the autophagy classifiers, as well as code to segment and extract single-cell images from entire fields of view up to WSIs (<https://github.com/MannLabs/SPARCSpy>). The image processing tools are now aggregated in scPortrait (<https://github.com/MannLabs/scPortrait>). Code to recreate the figures is available on GitHub (https://github.com/MannLabs/SPARCS_pub_figures).
- Any additional information required to reanalyze the data reported in this paper is available from the [lead contact](#) upon request.

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AUTHOR CONTRIBUTIONS

Conceptualization: N.A.S. and S.C.M.; formal analysis: N.A.S., S.C.M., and G.W.; funding acquisition: F.J.T., M.M., and V.H.; investigation: N.A.S.,

S.C.M., G.W., A.M., V.V., S.S., S.B.C., M.B., and H.H.; resources: H.H., H.L., F.J.T., M.M., and V.H.; software: N.A.S., S.C.M., and G.W.; visualization: N.A.S., S.C.M., and G.W.; writing—original draft: N.A.S., S.C.M., M.M., and V.H.; writing—review & editing: N.A.S., S.C.M., G.W., A.M., V.V., S.S., S.B.C., M.B., H.H., H.L., F.J.T., M.M., and V.H.

DECLARATION OF INTERESTS

N.A.S. consulted for Lamin Labs GmbH. S.C.M. consulted for Lamin Labs GmbH and is a current employee of Enocell Therapeutics. G.W. is a founder of Aplusia GmbH. S.S. is a current employee of Thermo Fisher Scientific. F.J.T. consults for Immunai, CytoReason, Genbio, Valinor Industries, Bioturing, and Phylo Inc. and has ownership interest in RN.AI Therapeutics, Dermagnostix, and Cellarity. M.M. is an indirect investor in Evosep Biosciences and a member of the advisory board of *Cell*.

DECLARATION OF GENERATIVE AI AND AI-ASSISTED TECHNOLOGIES IN THE WRITING PROCESS

During the preparation of this work, the authors used ChatGPT and Claude to edit text. After using these tools, the authors reviewed and edited the content as needed and take full responsibility for the content of the published article.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- [KEY RESOURCES TABLE](#)
- [EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS](#)
- [METHOD DETAILS](#)
 - Cell culture
 - Genome engineering
 - Immunofluorescence staining
 - Golgi compartment pH measurement
 - Laser microdissection
 - LC-MS phenotyping of SPARCS hits
 - CNN-based image classifier training
 - Segmentation of individual cells
 - SPARCS sample preparation and imaging of autophagy and STING trafficking
 - Genetic screening for autophagy regulators
 - Genetic screening for STING regulators
 - Immunoblotting
 - *In silico* perturbation modeling
- [QUANTIFICATION AND STATISTICAL ANALYSIS](#)

SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-LC3	Cell Signaling Technology	Cat# 3868S; RRID:AB_2137707
Anti-rabbit-HRP	Cell Signaling Technology	Cat# 7074S; RRID:AB_2099233
Anti-Actin-HRP	Santa Cruz Biotechnology	Cat# sc-47778 HRP; RRID:AB_2714189
GM130 (D6B1) XP® Rabbit mAb	Cell Signaling Technology	Cat# 12480S; RRID:AB_2797933
Goat anti-Rabbit IgG Alexa 647	Invitrogen	Cat# A-21245; RRID:AB_2535813
TGOLN2/TGN38 (E7W1N) Rabbit mAb	Cell Signaling Technology	Cat# 55727S; RRID not available
Phospho-TBK1/NAK (Ser172) (D52C2) XP® Rabbit mAb	Cell Signaling Technology	Cat# 5483S; RRID:AB_10693472
TBK1/NAK	Cell Signaling Technology	Cat# 3013S; RRID:AB_2199749
Phospho-IRF3 (Ser396) (4D4G) Rabbit mAb	Cell Signaling Technology	Cat# 4947S; RRID:AB_823547
IRF3 (D83B9) Rabbit mAb	Cell Signaling Technology	Cat# 4302S; RRID:AB_1904036
Phospho-STING (Ser366) (D7C3S) Rabbit mAb	Cell Signaling Technology	Cat# 19781S; RRID:AB_2737062
STING (D2P2F) Rabbit mAb	Cell Signaling Technology	Cat# 13647S; RRID:AB_2732796
Chemicals, peptides, and recombinant proteins		
Torin-1	Invivogen	inh-tor1
Monensin	Sigma-Aldrich	M5273-1G
Bafilomycin A1	Invivogen	tlrl-baf
Nigericin	Sigma-Aldrich	N7143-50MG
Brefeldin A	Sigma-Aldrich	B6542-5MG
Nocodazole	Sigma-Aldrich	M1404-10MG
cOMplete protease inhibitor cocktail	Sigma-Aldrich	11697498001
Wheat Germ Agglutinin Alexa 488	Thermo Fisher Scientific	W11261
Wheat Germ Agglutinin Alexa 647	Thermo Fisher Scientific	W32466
Alexa 488 Phalloidin	Cell Signaling Technology	8878S
Hoechst 33342	Sigma-Aldrich	B2261-25MG
PFA	Thermo Fisher Scientific	28908
DMEM	Thermo Fisher Scientific	41965062
RPMI	Thermo Fisher Scientific	21875091
Puromycin Dihydrochloride	Carl Roth	0240.4
Trypsin	Thermo Fisher Scientific	25300054
Pyruvate	Thermo Fisher Scientific	11360039
diABZI (Compound 3)	Selleck Chemicals	S8796
C53 (Compound 53)	Biomol	Cay37354-5
β-Estradiol	Sigma-Aldrich	E8875
Critical commercial assays		
MiSeq Reagent Kit v2, 300 Cycles	Illumina, San Diego, CA	Cat# MS-102-2002
Arcturus® PicoPure® DNA Extraction Kit	Thermo Fisher Scientific	KIT0103
Deposited Data		
SPARCS autophagosome and STING screen single-cell image data	This study	https://www.ebi.ac.uk/biostudies/bioimages/studies/S-BIAD3897
Mass spectrometry proteomics data	This study	PRIDE: PXD082653
Experimental models: Cell lines		
Human: U2OS	n/a	RRID:CVCL_0042
Human: HeLa	Gey et al. ¹⁰¹	RRID:CVCL_0030

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Human: CEBRA	This study	n/a
Oligonucleotides		
EI24_fwd: CACCGATACACTTACCGATAATTC	This study	n/a
EI24_rev: AAACGAATTATCGGTAAGTGTATC	This study	n/a
SEC24C_fwd: CACCGTTGTCGGGGGAGGGCCATA	This study	n/a
SEC24C_rev: AAACATATGGCCCTCCCCGACAAC	This study	n/a
ATG5_fwd: CACCGGCCATCAATCGGAAACTCA	This study	n/a
ATG5_rev: AAACGTAGTTTCCGATTGATGGCC	This study	n/a
screen_P5_0stagger: AATGATACGGCGACCA CCGAGATCTACACTCTTTCCCTACACGACGCTC TTCCGATCTTTGTGGAAGGACGAAACACCG	Doench et al. ⁵⁷	n/a
screen_P5_full_8stagger: AATGATACGGCGACCA GAGATCTACACTCTTTCCCTACACGACGCTCTTCCG ATCTGAAGACCCCTTGTGGAAGGACGAAACACCG	Doench et al. ⁵⁷	n/a
screen_P5_full_4stagger: AATGATACGGCGACCA CGAGATCTACACTCTTTCCCTACACGACGCTCTT CCGATCTCAACTTGTGGAAGGACGAAACACCG	Doench et al. ⁵⁷	n/a
screen_P5_full_2stagger: AATGATACGGCGACCA CGAGATCTACACTCTTTCCCTACACGACGCTCTT CGATCTGCTTGTGGAAGGACGAAACACCG	Doench et al. ⁵⁷	n/a
screen_P5_full_1stagger: AATGATACGGCGACCA ACCGAGATCTACACTCTTTCCCTACACGACGCT CTTCCGATCTCTTGTGGAAGGACGAAACACCG	Doench et al. ⁵⁷	n/a
screen_P5_full_3stagger: AATGATACGGCGACCA CGAGATCTACACTCTTTCCCTACACGACGCTCTT CGATCTAGCTTGTGGAAGGACGAAACACCG	Doench et al. ⁵⁷	n/a
screen_P5_full_6stagger: AATGATACGGCGACCA CGAGATCTACACTCTTTCCCTACACGACGCTCTT CGATCTTGACCTTGTGGAAGGACGAAACACCG	Doench et al. ⁵⁷	n/a
screen_P5_full_7stagger: AATGATACGGCGACCA AGATCTACACTCTTTCCCTACACGACGCTCTTCCGA TCTACGCAACTTGTGGAAGGACGAAACACCG	Doench et al. ⁵⁷	n/a
LentiGUIDE_P7_A1: CAAGCAGAAGACGGCATACG AGATCGGTTCAAGTGACTGGAGTTCAGACGTGTG CTCTCCGATCTTCTACTATTCTTTCCCTGCACTGT	Doench et al. ⁵⁷	n/a
LentiGUIDE_P7_A2: CAAGCAGAAGACGGCATACGA GATGCTGGATTGTGACTGGAGTTCAGACGTGTGCT CTTCCGATCTTCTACTATTCTTTCCCTGCACTGT	Doench et al. ⁵⁷	n/a
LentiGUIDE_P7_A3: CAAGCAGAAGACGGCATACG AGATTAACTCGGGTGACTGGAGTTCAGACGTGTGC TCTTCCGATCTTCTACTATTCTTTCCCTGCACTGT	Doench et al. ⁵⁷	n/a
LentiGUIDE_P7_A4: CAAGCAGAAGACGGCATACGAGA TTAACAGTTGTGACTGGAGTTCAGACGTGTGCTCTTC CGATCTTCTACTATTCTTTCCCTGCACTGT	Doench et al. ⁵⁷	n/a
LentiGUIDE_P7_A5: CAAGCAGAAGACGGCATACGAG ATATACTCAAGTGACTGGAGTTCAGACGTGTGCTCTT CCGATCTTCTACTATTCTTTCCCTGCACTGT	Doench et al. ⁵⁷	n/a
LentiGUIDE_P7_A6: CAAGCAGAAGACGGCATACG AGATGCTGAGAAGTGACTGGAGTTCAGACGTGTG CTCTCCGATCTTCTACTATTCTTTCCCTGCACTGT	Doench et al. ⁵⁷	n/a

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
LentiCRISPRv2_A1 CAAGCAGAAGACGGCATACGAGATCGGTTCAA GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC TCCAATTCCCACTCCTTTCAAGACCT	Doench et al. ⁵⁷	n/a
LentiCRISPRv2_A2 CAAGCAGAAGACGGCATACGAGATGCTGGATTGT GACTGGAGTTCAGACGTGTGCTCTTCCGATCTC CAATTCCTCACTCCTTTCAAGACCT	Doench et al. ⁵⁷	n/a
LentiCRISPRv2_A3 CAAGCAGAAGACGGCATACGAGATTAACGCG GGTGAAGTTCAGACGTGTGCTCTTCCGATC TCTCAATTCCCACTCCTTTCAAGACCT	Doench et al. ⁵⁷	n/a
LentiCRISPRv2_A4 CAAGCAGAAGACGGCATACGAGATTAACAGTT GTGACTGGAGTTCAGACGTGTGCTCTTCCGAT CTCCAATTCCCACTCCTTTCAAGACCT	Doench et al. ⁵⁷	n/a
LentiCRISPRv2_A5 CAAGCAGAAGACGGCATACGAGATATACTCAA GTGACTGGAGTTCAGACGTGTGCTCTTCCGAT CTCCAATTCCCACTCCTTTCAAGACCT	Doench et al. ⁵⁷	n/a
LentiCRISPRv2_A6 CAAGCAGAAGACGGCATACGAGATGCTGAGAA GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC TCCAATTCCCACTCCTTTCAAGACCT	Doench et al. ⁵⁷	n/a
LentiCRISPRv2_A7 CAAGCAGAAGACGGCATACGAGATATTGGAGGG TGACTGGAGTTCAGACGTGTGCTCTTCCGATC TCCAATTCCCACTCCTTTCAAGACCT	Doench et al. ⁵⁷	n/a
LentiCRISPRv2_A8 CAAGCAGAAGACGGCATACGAGATTAGTCTAAGT GACTGGAGTTCAGACGTGTGCTCTTCCGATCT CCAATTCCCACTCCTTTCAAGACCT	Doench et al. ⁵⁷	n/a
LentiCRISPRv2_A9 CAAGCAGAAGACGGCATACGAGATCGGTGA CCGTGACTGGAGTTCAGACGTGTGCTCTTCC GATCTCCAATTCCCACTCCTTTCAAGACCT	Doench et al. ⁵⁷	n/a
LentiCRISPRv2_A10 CAAGCAGAAGACGGCATACGAGATTACAGAGGG TGACTGGAGTTCAGACGTGTGCTCTTCCGATCT CCAATTCCCACTCCTTTCAAGACCT	Doench et al. ⁵⁷	n/a
hslFNB1_qPCR_fwd CAGCATCTGCTGGTTGAAGA	Gaidt et al. ¹⁰²	n/a
hslFNB1_qPCR_rev CATTACCTGAAGGCCAAGGA	Gaidt et al. ¹⁰²	n/a
hsCXCL10_qPCR_fwd TCTGAATCCAGAATCGAAGG	Gaidt et al. ¹⁰²	n/a
hsCXCL10_qPCR_rev CTCTGTGTGGTCCATCCTTG	Gaidt et al. ¹⁰²	n/a
GAPDH_qPCR_fwd GAGTCAACGGATTTGGTCGT	Gaidt et al. ¹⁰²	n/a
GAPDH_qPCR_rev GACAAGCTTCCCGTTCTCAG	Gaidt et al. ¹⁰²	n/a
GPR89B_crRNA_1 rArGrUrArArGrArCrArUrGrUrArGrUrGrUr ArUrGrUrUrUrArGrArGrCrUrArUrGrCrU	Integrated DNA Technologies	n/a

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
GPR89B_crRNA_2 rUrCrArCrUrCrCrArArUrCrArCrArCrCrArAr CrCrGrUrUrUrArGrArGrCrUrArUrGrCrU	Integrated DNA Technologies	n/a
Recombinant DNA		
Genome-wide human Brunello CRISPR library (LentiGUIDE-Puro)	Doench et al. ⁵⁷	Addgene-73178
Genome-wide human Brunello CRISPR library (LentiCRISPRv2)	Doench et al. ⁵⁷	Addgene-73179
pFUGW-mCherry-hsLC3	This study, Jahreiss et al. ¹⁰³	Addgene-40827
pFUGW-LckLip-mNeon	This study, Chertkova et al. ¹⁰⁴	Addgene-98821
pLenti6-MGAT-SEP-mRuby3	This study, Liu et al. ⁷⁵	Addgene-200937
pLenti6-GALT-SEP-mRuby3	This study, Liu et al. ⁷⁵	Addgene-200938
pBlast_hsGPR89B_FLAG	This study	n/a
pFUGW_hsSTING1_mNeon	This study	n/a
Lenti-Cas9-Blast	Sanjana et al. ¹⁰⁵	Addgene-52962
Software and algorithms		
py-lmd	This study	https://github.com/MannLabs/py-lmd
SPARCSpy	This study	https://github.com/MannLabs/SPARCSpy
SPARCStools	This study	https://github.com/MannLabs/SPARCStools
ASHLAR	Muhlich et al. ¹⁰⁶	https://github.com/labsyspharm/ashlar
Napari	Sofroniew et al. ¹⁰⁷	https://napari.org/stable/
Fiji	Schindelin et al. ¹⁰⁸	https://fiji.sc/
Prism 9.0	GraphPad, San Diego, CA	https://www.graphpad.com/scientific-software/prism/
DIA-NN 2.2.0	Demichev et al. ¹⁰⁹	https://github.com/vdemichev/diann
scPortrait	Mädler et al. ¹¹⁰	https://github.com/MannLabs/scPortrait
Scanpy	Wolf et al. ¹¹¹	https://scanpy.scverse.org/en/stable/
AlphaPeptTools	n/a	https://github.com/MannLabs/alphapepttools
Other		
PPS metal frame slides	MicroDissect	MDE5P80WK
Aurora Rapid column (5cm × 75μm, C18, 1.7μm particle size)	IonOpticks	AUR4-5075C18-CSI
384 well Protein LoBind plates	Eppendorf	0030624300
48-well plates (LMD sample collection)	Sarstedt	38.3923.500
Evotip Pure	Evosep	EV2018
Non-tissue culture treated 6-well plates	VWR	734-2777

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

The CEBRA N (C/EBP α -ER activated transdifferentiation system) cell line was generated via stable integration of a fusion construct of C/EBP α and the ligand-binding domain of the estrogen receptor (C/EBP α -ESR1LBD) into RCH-ACV cells using the piggyBac transposon system. RCH-ACV is a lymphoblastic leukemia cell line with chromosomal translocation t(1;19) and trisomy 8. Controlled nuclear translocation of the C/EBP α -ESR1LBD fusion protein, induced by β -estradiol, in the presence of interleukin-3 (IL-3) and macrophage colony-stimulating factor (M-CSF), induces the conversion of B-lineage cells to monocytes/macrophages while ensuring cell viability. The CEBRA N cell line carries a gene that confers resistance to nourseothricin.

METHOD DETAILS

Cell culture

Human female U2OS and HeLa cells were cultured in DMEM (Thermo Fisher Scientific #41965062) supplemented with 10 % fetal calf serum (FCS) (gibco #10270-106), penicillin/streptomycin (Thermo Fisher Scientific #15-140-122) and 1 mM sodium pyruvate (Thermo

Fisher Scientific # 11360039) and split every 2–3 days. U2OS and HeLa cells were split by incubating with 0.05% Trypsin-EDTA for 10 minutes at 37 °C after washing in PBS.

4 μ m PPS membrane slides (MicroDissect #MDE5P80WK) were sterilized for 30 minutes under the UV light of a cell culture hood with their cavity side down. Cells were then plated onto these slides cavity down in the three equally-sized wells of 4-well plates (Greiner Bio-One #96077307) with 5 mL DMEM per well.

CEBRA cells were cultured in RPMI (Thermo Fisher Scientific #21875091) supplemented with 10 % fetal calf serum (FCS) (gibco #10270-106), penicillin/streptomycin (Thermo Fisher Scientific #15-140-122) and 1 mM sodium pyruvate (Thermo Fisher Scientific #11360039) and split every 3–4 days. Transdifferentiation into monocytes was done with IL-3, MCSF and β -Estradiol as described previously,⁹⁶ with the modification that cells were differentiated in 6-well plates (VWR #734-2777) at a density of 1.4 million cells in 2 mL. Cells were detached on day 5 and plated into 96-well plates at a density of 100,000 cells in 100 μ L per well.

N/TERT-1 cells were cultured in a 1:2 mixture of Ham's F12 (Cat#21765029, Gibco) and Dulbecco's modified Eagle's medium (DMEM) (high glucose, no glutamine, no calcium; Cat#21068028, Gibco) supplemented with 1% minimum essential medium (MEM) nonessential amino acids (Cat#11140035, Gibco), 0.5% EpiLife defined growth supplement (produced in-house), 25 μ g/mL bovine pituitary extract (Cat#13028014, Gibco), 30 ng/mL EGF (Max Planck Institute of Biochemistry), 10 mM HEPES (Cat#H0887-100ML, Sigma-Aldrich), 2 mM GlutaMAX (Cat#35050038, Gibco), 0.1 mM CaCl_2 (Cat#C7902-500G, Sigma-Aldrich), and 100 U/ml penicillin-streptomycin (Cat#15140122, Gibco).

All cells were cultured in a humidified incubator at 37°C with 5% CO_2 .

Genome engineering

U2OS cells stably expressing Cas9, mCherry-LC3 (the original plasmid was a gift from David Rubinsztein (Addgene plasmid #40827,¹⁰³)) and mNeon tagged N-terminally with the lipidation sequence of Lck (LckLip-mNeon, the original plasmid was a gift from Dorus Gadella (Addgene plasmid #98821,¹⁰⁴)) were generated by lentiviral transduction; HeLa hsSTING1-mNeonGreen cells were also generated by lentiviral transduction. Briefly, HEK-293T cells were transfected with transfer plasmids for Cas9 or mCherry-LC3 and 3rd generation lentiviral particle production plasmids pMDLg and pRSV as well as a VSV G-protein pseudotyping plasmid 18 h after plating. 8 h later, the medium was exchanged and cells were washed once in PBS (gibco #14190169). After 48 h supernatants containing viral particles were harvested and transferred onto target cells plated 18 h before. 48 h later target cells were washed. Cells were selected for Blasticidin resistance with 10 μ g/mL Blasticidin (Thermo Fisher Scientific #A1113903) or FACS-sorted for high fluorescent protein expression and single clones generated by limiting dilution cloning. For mCherry-LC3 U2OS cells, a bright clone with a visible reaction to 600 nM Torin-1 (invivogen #inh-tor1) was selected, expanded and used for all experiments. Lentiviral particles for the expression of individual sgRNAs from LentiGUIDE-Puro were generated analogously. Cells were selected for sgRNA expression with 5 μ g/mL puromycin (Carl Roth #0240.4) for 48 h. Of note, the cell line used for the autophagy screens did not yet stably express Cas9 but was instead transduced with LentiCRISPRv2, a vector driving expression of both Cas9 and an sgRNA. HeLa hsSTING1-mNeon also contain pFUGW-mCherry-hsLC3 and LentiCas9-Blast. A bright clone of these HeLa hsSTING1-mNeon cells with visible STING trafficking upon diABZI stimulation was selected, expanded and used for all experiments.

Individual knockout cell lines were generated with LentiGUIDE-Puro as described above or using the CRISPR/Cas9 system with ribonucleoprotein (RNP) delivery. RNPs were generated by annealing 1:1 molar ratio of tracrRNA and crRNA at 95 °C for 5 min, followed by stepwise (-1 °C/35 sec) decrease to 22 °C. To each gRNA complex (100 pmol) NLS-Cas9 (40 pmol) was added and incubated at room temperature for 15 min. CEBRA cells were nucleofected using the SG Cell Line 96-well Nucleofector kit (Lonza). 5×10^5 cells were re-suspended in 20 μ L of SG Cell Line Nucleofector Solution. The Cas9/RNP assembly was added, and the cells were nucleofected on a 4D-Nucleofector (Lonza) using the program EH-100. N/TERT-1 and HeLa cells were nucleofected using the P3 Primary Cell Line 96-well Nucleofector kit (Lonza). 1×10^6 cells were re-suspended in 20 μ L of P3 Cell Line Nucleofector Solution. The Cas9/RNP assembly was added, and the cells were nucleofected on a 4D-Nucleofector (Lonza) using the program DS-138. Subsequently, cells were transferred to pre-warmed culture medium. Cells were rested for 48 h, followed by seeding at limiting dilutions to obtain monoclonal populations.

Immunofluorescence staining

After stimulation with 10 μ M BFA for 1 h or 5 μ g/mL Nocodazole for 30 minutes, PPS slides were washed 1 $\times$ in PBS in a Coplin jar and fixed for 10 minutes at room temperature in 4 % MeOH-free PFA in PBS in 4-well plates before being washed 3 $\times$ in PBS and then dried by centrifuging at 3,400 g for 1 minute. The fixed slides were washed once in PBS before permeabilization with 0.1 % Triton-X 100 in PBS for 10 minutes. After washing 2 $\times$ in PBS, slides were blocked with 5% BSA in PBS for 10 minutes. Following blocking, slides were incubated with primary antibody at 1:200 dilution in PBS at 4 °C overnight. After washing 3 $\times$ in PBS, slides were incubated with secondary antibody at 1:400 dilution in PBS for 1 h at room temperature. Following 3 $\times$ washing in PBS, slides were incubated with Alexa 488 Phalloidin at 330 nM for 15 minutes at room temperature. After washing 2 $\times$ in PBS, slides were stained with 10 μ g/mL Hoechst-33342 in PBS at room temperature for 30 minutes. After washing 3 $\times$ in PBS, slides were dried in a centrifuge at 3,400 g for 1 minute. Slides were imaged cavity-up on inverted microscopes, with cells directly above the objective lens.

Golgi compartment pH measurement

Ratiometric fluorescent pH-sensors targeted to the cis- and medial Golgi (based on Addgene #200937) or the trans-Golgi network (based on Addgene #200938) were stably expressed in HeLa cells. Cells were FACS sorted for high expression of the sensors. A

polyclonal *GPR89A*; *GPR89B* knockout was made for each sensor. Immediately following treatment with bafilomycin A1 (1 μ M) or monensin (50 μ M), cells were imaged on a Leica Dmi8 fluorescence microscope. Calibration curves were recorded by incubating cells in a pH-adjusted buffer (125 mM KCl, 20 mM NaCl, 0.5 mM MgCl₂, 0.2 mM EGTA) with 20 mM HEPES (pH 7.0, 7.5) or 20 mM MES (pH 5.5, 6.0, 6.5). Buffer pH was adjusted with HCl or KOH. The buffer was supplemented with 10 μ M monensin and 10 μ M Nigericin before addition to cells.

Laser microdissection

We plated cells onto the non-cavity side of polyphenylene sulfide (PPS) metal frame membrane slides. Before cell plating, landmark crosses were cut into membranes for image registration between imaging and laser microdissection microscopes. Cutting paths for laser microdissection of landmark crosses and selected cells were generated using our open-source Python library py-lmd (<https://github.com/MannLabs/py-lmd>) with the configurations specified in the “autophagy screen config”, “sting screen nucleus config” and “sting screen whole cell config” files, respectively. By minimizing autofocus time and optimizing the trade-off between laser speed and accuracy, compressing the cutting path shape (implemented in py-lmd) and leveraging the fact that it is sufficient to isolate nuclei to determine a cell’s CRISPR perturbation by sequencing, we achieved fully autonomous excision at a speed of 1,000 nuclei per hour. For nuclear excision, nuclear borders were used to define cutting paths, and each shape was dilated to ensure that the cutting line did not intersect or damage the nucleus. In contrast, for whole cell excision, cytosolic borders were used instead, and shape dilation was kept to a minimum to reduce contamination from neighboring cells. Laser microdissection was carried out on a Leica LMD7 at 40 $\times$ magnification using the software version 8.3.0.8275 for the autophagy screen and 8.5 for the STING screen respectively. The microscope was equipped with the Okolab LMD climate chamber (H201-ENCLOSURE-LMD and H201-LEICA-LMD) to ensure stable temperatures throughout the cutting process. Slides were equilibrated in the microscope to 34.5 $^{\circ}$ C before cutting to ensure focus stability. After reference point alignment to the landmark crosses, cutting contours were imported from the XML files generated with py-lmd and excised using screen-specific settings. For the autophagy screen, cuts were performed at power 60, aperture 1, speed 100, head current 46 %–51 %, pulse frequency 1128 and offset 185 with autofocus adjustment every 30 shapes. For STING screen nucleus excision, settings were power 60, aperture 4, speed 100, middle pulse count 1, final pulse -6, head current 43 %, pulse frequency 2273, and offset 201, with autofocus adjustment every 4 shapes. For STING screen whole-cell excision, settings were power 55, aperture 3, speed 99, middle pulse count 1, final pulse 0, head current 44 %, pulse frequency 2275, and offset 188, with autofocus adjustment performed before every shape. Excised shapes were collected in 48-well plates (Sarstedt #38.3923.500) for downstream gRNA sequencing (500–2,000 nuclei per well) or 384-well Proteint LoBind plates (Eppendorf #0030624300) for mass-spectrometry analysis (40–60 cells per well). During cutting a custom-built wind protection was used around the collection plate to ensure collection of excised shapes into the center of the well and prevent wind disturbances. After cutting, samples were stored at 4 $^{\circ}$ C before lysis and library generation or mass-spectrometry sample preparation.

LC-MS phenotyping of SPARCS hits

We generated embeddings of intracellular STING distribution in screen cells from different classification bins by forward-passing single-cell images through ImageNet-trained ConvNeXt.¹¹² Embedding features were standardized prior to downstream analysis. To enable batching of morphologically similar cells for laser microdissection while enforcing cluster size constraints, clustering was formulated as a constrained mixed-integer optimization problem. Embeddings were reduced to 20 principal components, and cells were partitioned into $K = N/50$ clusters by minimizing the sum of squared distances in PCA space. Constraints required that each cell be assigned to exactly one cluster and that each cluster contain between 40 and 60 cells. The optimization was solved using the CBC solver implemented in PuLP (<https://github.com/coin-or/pulp>). For the analyzed dataset ($N = 2,128$), this yielded 43 clusters satisfying the specified size constraints.

Preparation of the microdissected samples for LC-MS was performed in 384-well plates in an automated fashion utilizing a robotic liquid handling system (Agilent Bravo). Collection plates were centrifuged (2,000 g, 2 min) and each well was rinsed with 28 μ L of 100% acetonitrile, then vacuum-dried at 45 $^{\circ}$ C for 20 minutes to retain isolated cells at the bottom of the well. A lysis buffer consisting of 0.013% DDM in 60 mM TEAB (pH 8.5) was added to each well (6 μ L per well). Plates were sealed using PCR foil and incubated at 95 $^{\circ}$ C for 30 minutes in a PCR cycler with a lid temperature of 110 $^{\circ}$ C. Subsequently, 1 μ L of 80% acetonitrile was added and the samples were heated at 75 $^{\circ}$ C for 30 minutes. Once cooled, enzymatic digestion was initiated by adding 1 μ L of a mixture of LysC (4 ng/ μ L) and trypsin (6 ng/ μ L) prepared in 60 mM TEAB buffer, followed by overnight incubation ($\sim$ 18 h) at 37 $^{\circ}$ C. Digestion was stopped by acidification with 1 μ L of 10% trifluoroacetic acid. The resulting digested samples were loaded on Evotip Pure tips (Evosep #EV2018), according to the manufacturer’s instructions.

The samples were chromatographically separated using an Evosep One running the Whisper 80 samples per day (16-minute-long) chromatographic gradient on an Aurora Rapid column (IonOpticks, 5 cm $\times$ 75 μ m, C18, 1.7 μ m particle size, #AUR4-5075C18-CSI) at 55 $^{\circ}$ C and 1,900 V and subsequently measured on a Thermo Scientific Orbitrap Astral MS coupled with the FAIMS Pro Interface operating at a single compensation voltage of -40 V. The MS instrument settings had a 240K MS1 resolution, with a variable MS2 window approach targeting deepest proteomic coverage. Specifically, we used 100 windows spanning the range from 380 to 980 m/z and a maximum injection time of 10 ms.

Raw data files were searched, using DIA-NN 2.2.0¹⁰⁹ in library-free mode, enabling both “FASTA digest for library-free search / library generation” and “Deep learning-based spectra, RTs and IMs prediction”. The FASTA file used was UP000005640_9606 (retrieved from UniProt, July 2021). An additional contaminants file was used from Frankenfield et al.¹¹³ The rest of the settings used were as follows: up to 1 missed cleavage, up to 1 variable modification (N-term M excision, Ac(N-term) and Ox(M)), 7–30 peptide length range, 2–4 precursor charge range, 300–1800 precursor m/z range, 300–1,800 fragment ion m/z range, precursor FDR 1%, peptidoform scoring, genes for protein inference, NN (cross-validated) mode for neural network classifier, QuantUMS (high precision) for quantification strategy and RT-dependent for cross-run normalization. The “MBR”, Unrelated Runs and Protein inference boxes were also checked.

Raw DIA-NN gene-level protein intensities were loaded into Python using alphapeptools (<https://github.com/MannLabs/alphapeptools>) with second-search false discovery rate (FDR) filtering enabled. Proteins containing exclusively missing values across all samples were removed. Sample-level quality control was performed on log₂-transformed intensities using robust median absolute deviation (MAD)-based filtering. Samples with median log₂ intensities deviating by more than 3 MAD from the global median, or with summed log₁₀ intensities exceeding 6 MAD from the global median, were excluded from downstream analyses. Following QC filtering, protein intensities were normalized by total-median normalization on linear-scale intensities and subsequently transformed to log₂ space.

Proteins detected in fewer than two samples within both the hit and control populations were excluded. In addition, keratin proteins were removed as likely contaminants. Missing values were imputed using Gaussian-based imputation on the log₂-normalized intensity matrix.

Differential expression analysis was performed on the Gaussian-imputed log₂-normalized data. Proteins were compared between hit (threshold > 0.99) and reference (threshold ≤ 0.01) groups using two-sided Welch’s t-tests in scipy.¹¹⁴ Log₂ fold changes were calculated as the difference between group means in log₂ intensity space. P-values were adjusted for multiple testing using the Benjamini–Hochberg false discovery rate (FDR) procedure. Gene-wise fold-change estimates derived from the pooled hit-versus-reference comparison were subsequently used as MS-derived signatures for *in silico* perturbation modeling.

CNN-based image classifier training

For classification of autophagy and Golgi morphology, neural networks with 9 randomly initialized convolutional layers and 3 (classifiers 1.0 & P) or 4 (classifiers 2.0, 2.1, Golgi-Noco, Golgi-BFA) linear layers were trained to classify segmented single-cell images (Table S1).

The training datasets for the Golgi classifiers consisted of 3 (Noco) or 4 (BFA) biological replicates with one treated and one untreated slide each. 150k cells from each slide were used for training the classifiers. 20 k cells from each slide were kept separate for validation during training and testing after training, respectively.

The training datasets for classification into autophagy-on or autophagy-off cells were based on several biological replicates of mCherry-LC3-expressing U2OS cells with and without autophagosomes. The autophagy-off class consisted of images from unstimulated wildtype cells (pre-filtered to remove cells showing spontaneous autophagosome formation for 2.0 and 2.1) and two different ATG5^{-/-} clones. Where applicable, pre-filtering was performed with classifier P. The autophagy-on class consisted of single-cell images from stimulated wildtype cells, where applicable pre-filtered with classifier P to remove non-responding cells. To increase variability captured in the training data, the training slides were plated at an angle to include varying cell densities on one slide. 500 k, 1 million or 1.2 million single-cell images respectively were randomly selected from each class for training while ensuring balanced sampling from each slide. An additional 50 k cells from each class were used for testing and validation during training. Training data were augmented by Gaussian blur, addition of Gaussian noise and random rotations in 90° steps. Training was performed using stochastic gradient descent with a learning rate of 1×10^{-3} . Gradient clipping was set to 0.5. Training was performed over a total of 20, 30 or 40 epochs. Classifier performance was tested on a biologically independent set of unstimulated wildtype cells, Torin-1-stimulated wildtype cells and ATG5^{-/-} cells.

To classify subcellular STING localization based on single-cell images, we fine-tuned the ImageNet-pretrained CNN ConvNeXt¹¹² (<https://huggingface.co/facebook/convnext-large-224-22k>) with one additional linear layer as a four-class classifier for six epochs. Single-channel input images were triplicated to conform to the three-channel input expected by ConvNeXt. Training data with four class labels corresponding to ER, ERGIC, TGN and lysosome localization of STING were generated by treating hsSTING1-mNeon-expressing HeLa cells with diABZI for 0 h, 0.5 h, 1 h and 2 h before 40× imaging on an Opera Phenix microscope in spinning disk mode. Single-cell image datasets were generated with scPortrait¹¹⁰ (<https://github.com/MannLabs/scPortrait>).

Models were built and trained using PyTorch.¹¹⁵

Segmentation of individual cells

Images were flat-field corrected during image acquisition using the PerkinElmer Harmony software (v4.9) and intensity rescaled to the 0.01 and 0.99 quantile. Extremely bright regions (pixel values greater than 40,000) were set to 0 before determining the normalization quantiles. Stitching of image tiles was performed using the ashlar Python API¹⁰⁶ in our open-source Python library SPARCStools (<https://github.com/MannLabs/SPARCStools>) for the autophagy screen and scPortrait (<https://github.com/MannLabs/scPortrait>)¹¹⁰ for the STING screen.

Stitched whole-slide images were segmented using our open-source SPARCSpy Python library (<https://github.com/MannLabs/SPARCSpy>) for the autophagy screen or scPortrait¹¹⁰ for the STING screen with the parameters defined in “config_screen_autophagy”, “config_training_autophagy”, “config_training_Golgi”, or “config_screen_STING” respectively. For the autophagy data a nucleus segmentation mask was generated using a local median thresholding approach and the cytosol segmentation mask was calculated using fast marching from nuclear centroids with WGA staining as a potential map. The Golgi data were segmented based on nucleus and Phalloidin staining using Cellpose.¹¹⁶ The STING data were segmented based on nucleus and WGA staining using Cellpose.¹¹⁶ Nucleus and cytosol masks were matched on a per-cell basis, requiring at least 95 % overlap of the nucleus mask with the corresponding cytosol mask; masks not meeting this threshold were discarded.

Single-cell images were extracted based on cytosolic segmentation masks. For the autophagy screen, the masked area was extended using a Gaussian filter with a sigma of 5 to extract information from each of the imaged channels and saved to hdf5 files as individual 128 × 128 px images. For the STING screen, single-cell images with 320 × 320 px were extracted with scPortrait and saved as h5sc files.¹¹⁰

SPARCS sample preparation and imaging of autophagy and STING trafficking

After stimulation with 600 nmol/L Torin-1 for autophagy or with 250 nmol/L diABZI (Selleck Chemicals #S8796) for STING trafficking, PPS slides were washed 1x in PBS in a Coplin jar and then stained with 10 µg/mL WGA-Alexa488 (Thermo Fisher Scientific #W11261) for the autophagy screen or WGA-Alexa647 (Thermo Fisher Scientific #W32466) for the STING screen in PBS for 10 minutes at 37 °C. After washing 1 × with PBS, slides were fixed for 10 minutes at room temperature in 4 % MeOH-free PFA (Thermo Fisher Scientific #28908) in PBS in 4-well plates. After washing 3 × in PBS, slides were stained with 10 µg/mL Hoechst-33342 (Sigma Aldrich #B2261-25MG) in PBS at 37 °C for at least 30 minutes. After washing 3 × in PBS, slides were dried in a centrifuge at 3,400 g for 1 minute. Cells in microwell slides (ibidi #80826) and plates were stained according to the same protocol and imaged in PBS. Imaging was done on a Nikon Eclipse Ti2 spinning disk confocal microscope or an Opera Phenix high-content imager as indicated. For follow-up experiments assessing STING trafficking and activation, cells were treated with 500 nmol/L diABZI, 10 µmol/L C53, or 50 µmol/L monensin, alone or in the combinations indicated in the figure legends.

Genetic screening for autophagy regulators

We conducted our screen in mCherry-LC3-expressing U2OS cells using the Brunello human CRISPR KO library in the LentiCRISPRv2 backbone. The Brunello library was a gift from David Root and John Doench (Addgene #73179) and amplified according to their protocol.⁵⁷ U2OS cells were transduced with lentiviral particles produced as described above at an MOI of approximately 0.2. After 48 h, successfully transduced cells were selected with 5 µg/mL puromycin for two days and then expanded for three days. We then plated 50 million cells on a total of 109 slides in 4-well plates, and in addition included unstimulated and wildtype controls on separate slides with every screening batch for classifier training. The day after plating, cells were stimulated with 600 nmol/L Torin-1 for 14 h. Slides were then prepared for microscopy as described above and imaged on an Opera Phenix high content imager at 20 × magnification. After segmentation, cells were classified into autophagy-on and autophagy-off (Table S1). We observed that test set scores differed for unstimulated wildtype cells and ATG5^{-/-} cells, indicating that our classifier assigned different scores to different autophagy phenotypes. We therefore divided all hits (classifier score > 0.98) into six bins by their post-softmax classification scores as follows (Figures 3C and S7C): Bin #1 represented a cutoff (0.99999) at which we found ATG5^{-/-} to be strongly enriched in our test dataset. Bin #2 corresponded to the respective cutoff for unstimulated wildtype cells (0.999). Bins 3–6 contained the remaining potential hits with a roughly equal number of cells per bin. Multiple pools of nuclei were collected in separate wells per bin to avoid sequencing large numbers of excised nuclei in a single reaction.

Where applicable, slides were stored at -20 °C and brought to 4 °C the day before laser microdissection. Cells from each bin were excised into multiple wells. Nuclei were then lysed in 48-well plates using the Arcturus PicoPure™ DNA extraction kit (Thermo Fisher Scientific #KIT0103). 120 µL of lysis buffer was added to each well and incubated at 65 °C for 4 h. Proteinase K was inactivated at 95 °C for 15 minutes. Cooled samples were transferred to PCR tubes and the emptied wells were rinsed with 40 µL of ddH₂O. Amplification of sgRNAs was performed as described previously¹¹⁷ but in a single-step PCR⁵⁷ over 36 cycles with no added water. Sequencing was performed on an Illumina NextSeq with 500 reads per nucleus on average. An sgRNA read count table was generated for each sequencing library. Low quality sgRNAs were removed by applying a minimum number of reads per sgRNA threshold that was set based on the distribution of read counts per sgRNA in the sample. The non-targeting sgRNA with the sequence TACGTCATTAAGAGTTCAAC was excluded from sequencing results of approximately 40 % of cells from bins 3–6 of batch 2 of the genome-wide screen due to a contamination of the sequencing library leading to abnormally high read counts of this specific sequence. sgRNA read counts were divided by the sum of all sgRNA read counts in the sequencing library (“normalized counts”). For further analysis only sgRNAs with at least a fraction of reads corresponding to a single cell per well were used. sgRNA read fractions of individual wells were then aggregated per bin by multiplying with the fraction of excised cells in that well over all excised cells in the bin. The per-bin aggregated sequencing results were used for all further data analysis. Enrichment values were determined by normalizing the aggregated fraction of reads per sgRNA to the fraction of reads per sgRNA in the input cell library. The gene set for the identification of known autophagy regulators is based on the union of the gene ontology terms “Autophagy” (GO:0006914), “Autophagy of mitochondrion” (GO:0000422), “Autophagosome assembly” (GO:0000045) and “Nucleophagy” (GO:0044804). The final list is available in Table S4.

Genetic screening for STING regulators

We conducted our screen in Cas9, hsSTING1-mNeon, mCherry-LC3-expressing HeLa cells using the Brunello human CRISPR KO library in the LentiGUIDE-Puro backbone. The Brunello library was a gift from David Root and John Doench (Addgene #73178) and amplified according to their protocol.⁵⁷ HeLa cells were transduced with lentiviral particles produced as described above at an MOI of approximately 0.2. After 48 h, successfully transduced cells were selected with 5 μ g/mL puromycin for two days and then expanded for three days. We then plated 99.75 million cells on a total of 105 slides in 4-well plates in 6mL per well, and in addition included unstimulated and wildtype controls on separate slides with every screening batch for classifier training. 16 h after plating, cells were stimulated with 250 nmol/L diABZI for 2 h. Slides were then prepared for microscopy as described above and imaged on an Opera Phenix high content imager at 40 $\times$ magnification. Where applicable slides were stored at -20 $^{\circ}$ C and brought to 4 $^{\circ}$ C the day before laser microdissection. Excised nuclei were then lysed in 48-well plates using the Arcturus PicoPureTM DNA extraction kit (Thermo Fisher Scientific #KIT0103). 120 μ L of lysis buffer was added to each well and incubated at 65 $^{\circ}$ C for 4 h. Proteinase K was inactivated at 95 $^{\circ}$ C for 15 minutes. Cooled samples were transferred to PCR tubes and the emptied wells were rinsed with 40 μ L of ddH₂O. Amplification of sgRNAs was performed as described previously¹¹⁷ but in a single-step PCR⁵⁷ over 36 cycles with no added water. Sequencing was performed on an Illumina NextSeq with 500 reads per nucleus on average.

Immunoblotting

20,000 U2OS cells were plated per 96-well. 18 h after plating, cells were stimulated with 600 nmol/L Torin-1 and 0.1 μ M Bafilomycin A1 (invivogen #tlrl-baf) and then harvested in 1 $\times$ Lämmli buffer. 3 wells were pooled per condition. Lysates were boiled at 95 $^{\circ}$ C for 5 min. and then run on 16 % TRIS-glycine polyacrylamide gels (Thermo Fisher Scientific #XP00165BOX). 100,000 differentiated CEBRA cells were plated per 96-well and stimulated as indicated. Up to five wells were pooled per condition. Cells were harvested in DISC buffer (150 mM NaCl, 50 mM Tris pH 7.5, 10% glycerol, 1% Triton X-100) with cOmplete protease inhibitor cocktail. Protein concentrations were determined via BCA assay, samples were boiled in 1 $\times$ Lämmli buffer (added as 6 $\times$ concentrate), and 5 μ g were loaded per lane of a 12 % TRIS-glycine polyacrylamide gel (Thermo Fisher Scientific #XP00125BOX). Blotting onto 0.45 μ m nitrocellulose membrane (Sigma Aldrich #GE10600002) was done for 90 minutes at 100V. Membranes were blocked in 5 % milk in PBST for 1 h and incubated with primary antibody at 4 $^{\circ}$ C overnight. After washing 3 $\times$ in PBST for a total of 15 min. membranes were incubated with HRP-labeled secondary antibody for 2 h at room temperature. After washing 3 $\times$ in PBST for a total of 15 min. membranes were covered in luminescent HRP substrate (Merck #WBLUF0500) and immediately imaged.

In silico perturbation modeling

Reference single-cell HeLa transcriptomes from two datasets were downloaded from GEO (GSM3713084 and GSM3713085)¹¹⁸ and pre-processed in scanpy.¹¹¹ Genes detected in fewer than 10 cells were removed, and cells were filtered by gene and count thresholds, hemoglobin fraction, and ribosomal read fraction. Doublets were identified with Scrublet using the dataset source as the batch key and removed. The count matrix was normalized to a target sum of 10,000 and log_{1p} normalized. After log-normalization, principal component analysis (PCA) was performed on scaled highly variable genes to identify low-quality or outlier cells. Cells with extreme coordinates in PCA space were removed using manual thresholds on specific principal components. In total, 150 cells from each dataset were used for downstream analysis.

Differential protein abundances between STING screen hits and non-hits (STING classifier threshold ≤ 0.01) were measured by mass spectrometry (MS) proteomics as described above. Reference transcriptomes were subset to genes encoding proteins measured in this proteomics dataset. To simulate STING screen hit single-cell transcriptomes, protein abundance changes between STING screen hits and non-hits were multiplied with reference single-cell transcriptomes, matching transcripts to the proteins they encode. Control transcriptomes were generated by 10,000 random permutations of gene labels within the MS-derived fold-change signature before multiplication with the reference transcriptomes. Simulated and reference transcriptomes were renormalized to a target sum of 10,000 counts per cell. Simulated and reference transcriptomes were encoded using the pretrained STATE Embedding (SE) model (<https://huggingface.co/arcinstitute/SE-600M>).⁸⁷

Genetic perturbation effects were then modeled with the STATE Transition (ST) model⁸⁷ using the per-cell SE inputs. For each reference cell, the SE embedding of the unmodified base cell state was passed to the pretrained ST model (<https://huggingface.co/arcinstitute/ST-SE-Replogle/tree/main/zeroshot/hepg2>) to predict the gene expression shift induced by all 2,023 genetic perturbations the ST model was trained on. In parallel, the SE embeddings of simulated single-cell profiles generated from the observed and gene-label-permuted hit DE signatures were passed to the ST model together with the non-targeting control perturbation. Predicted outputs were compared in scaled HVG space after subtracting the non-targeting reference cell state to center all embeddings and generate a vector space representing gene expression shifts relative to the base cell state. For each candidate genetic perturbation, the shift from the base state predicted by ST was compared to the shift from the base state induced by the observed MS-derived signature and to shifts induced by permuted MS signatures using cosine distance. Cosine distances were then converted to cosine similarities, and each genetic perturbation was scored by multiplying its observed similarity to the MS-derived hit DE gene signature (expected to be high for *in silico* screen hits) with the negative log fraction of permuted signatures to which the genetic perturbation was more similar than to the observed MS-derived hit DE gene signature (expected to be high for *in silico* screen hits). Candidate genes were ranked within each cell by this score.

To identify robust candidate genes across cells and datasets, per-cell perturbation rankings were aggregated across all evaluated cells. For each perturbation, the median rank across cells and the fraction of cells in which the perturbation ranked within the top 5% of all tested perturbations were calculated. These two metrics were combined to prioritize perturbations that consistently reproduced the MS-associated state shift across cellular backgrounds while remaining distinct from shifts induced by permuted controls. Statistical significance of aggregated perturbation rankings was assessed using an empirical null model based on randomized within-cell rank permutations. For each permutation, perturbation ranks were shuffled independently within each cell. Empirical p-values were calculated for the median rank and top 5% recurrence metrics by comparing observed values to the null distribution generated across permutations. A conservative combined empirical p-value was defined as the maximum of the median-rank and top-5%-recurrence empirical p-values. False discovery rates (FDRs) were subsequently estimated across all tested perturbations using the Benjamini–Hochberg procedure applied to the combined empirical p-values.

QUANTIFICATION AND STATISTICAL ANALYSIS

The statistical details of experiments can be found in the respective figure legends, including the statistical tests used, exact values of n and what n represents.