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Preview

Unveiling the hidden network of STING's subcellular regulation

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A new study deconvolutes the systems-level control of the cGAS-STING pathway and identifies many novel regulators of STING biology. This was made possible by optical pooled screening (OPS), which enables high-dimensional imaging of millions of gene-edited cells, showcasing the future of CRISPR screening.

The cyclic GMP-AMP synthase (cGAS)-stimulator of interferon genes (STING) pathway is a pivotal component of the innate immune system.[1,2] cGAS functions as a sentinel that detects both exogenous and endogenous DNA.[2] Upon activation, cGAS catalyzes the synthesis of cyclic GMP-AMP (cGAMP), a second messenger that engages STING. This triggers a cascade of immune responses, notably induction of type I interferon (IFN-I) and proinflammatory cytokines.[3] These processes are critical for host defense against pathogens but also involved in the pathophysiology of cancer and autoimmune disorders.

Owing to its potency, cGAS-STING signaling is intricately regulated at multiple levels, including by subcellular trafficking.[4] Once activated on the endoplasmic reticulum (ER) membrane,[2] STING translocates from the ER to the ER-Golgi intermediate compartment (ERGIC) and the Golgi via COPII vesicles. At the Golgi, STING recruits TBK1 through its C-terminal tail and is phosphorylated by TBK1. Additionally, STING undergoes palmitoylation, which facilitates its assembly into a signalosome and the recruitment of IRF3 for phosphorylation by TBK1. Phosphorylated IRF3 (p-IRF3) dimerizes and translocates to the nucleus to initiate the type I interferon response. STING also activates NF-κB through underdefined mechanisms. The regulation of STING trafficking extends beyond the Golgi. The adaptor protein complex-1 (AP-1) binds to STING upon STING phosphorylation, sorting it into clathrin-coated vesicles destined for endolysosomes.[4] After transiting from the Golgi, STING is ubiquitinated by UBE2N in the endosomes, recruiting the endosomal sorting complex required for transport (ESCRT). The ESCRT is crucial for directing STING toward lysosomal degradation and resolving its activity. The lysosomal protein Niemann-Pick C1 (NPC1) interacts directly with STING to mediate its recruitment to lysosomes. Additionally, STING engages with autophagy pathways, contributing to its degradation through ERGIC.[3] Therefore, precise trafficking of STING, and co-localization with specific proteins, is critical to its function, and dysregulation of these processes is linked to immune diseases, necessitating thorough investigation of how STING's journey through the cell is controlled.

CRISPR screening has revolutionized the search for genes contributing to different biological processes. However, typical pooled CRISPR screens, which readout gRNA frequency, provide limited phenotyping and low-content information about each gene. To overcome this limitation, techniques have been developed to permit transcriptomic,[5] proteomic,[6] and optical[7] analysis of single cells within a pooled CRISPR screen, which enable more extensive gene analysis.

To address the challenge of deconvoluting the complex control of STING regulation, including identification of the genes contributing to each step of STING's trafficking, Gentili et al.[7] utilized optical pooled screening (OPS),[8] a powerful approach to CRISPR genomics in which RNA barcodes and cellular markers are detected in single cells by microscopy imaging (Figure 1A). It is akin to a merge of traditional high-content screening and pooled CRISPR screening; it is like functional genomics on steroids. In an initial screen, a genome-wide CRISPR library was introduced into HeLa cells engineered to express a fluorescently labeled STING. The cells were stained for markers of the nucleus (DAPI), Golgi apparatus (GM130), endolysosomes (CD63), and an autophagy receptor (p62) to visualize the location of STING following activation by cGAMP treatment of the cells. Along with imaging STING's positioning, *in situ* sequencing was performed to determine the specific barcode, and thus gene knockout (KO), that each cell carried. This enabled the location or mislocation of STING to be assessed in every gene KO cell, along with thousands of other features extracted from the cells. Impressively, they performed this high-dimensional analysis on 45 million cells, permitting screening of the whole gRNA library and providing a robust dataset for analysis.

Using the different data features, they developed three linear support vector machine (SVM) classifiers—corresponding to perturbed, unstimulated, and enhanced stimulated cell states—which identified 695 gene hits. The results were integrated with data from previous non-OPS-based genome-wide CRISPR screens of STING regulators.[9] By employing meta-analysis by information content (MAIC), they compiled a consensus list of 1,225 genes that influence STING activity in at least two separate screens. To further elucidate the biological functions of these consensus genes, they conducted unsupervised

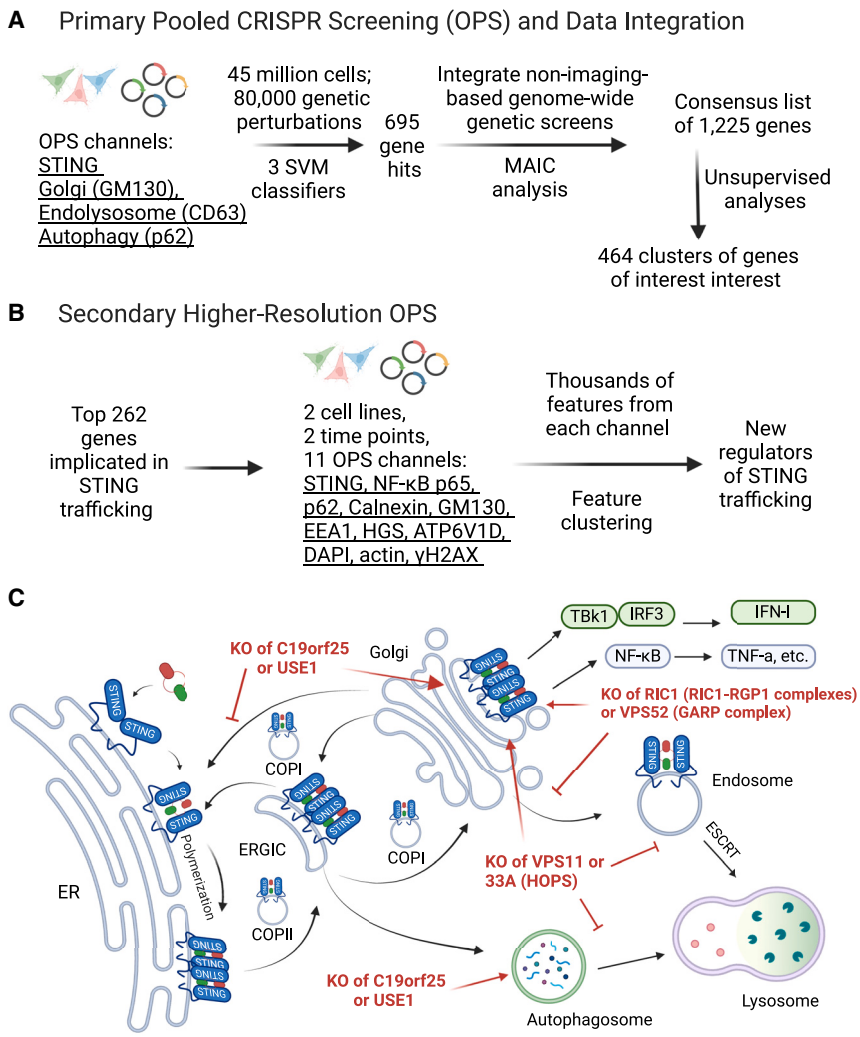

Figure 1. Genome-wide optical pooled screening (OPS) of STING trafficking regulators

(A) Primary OPS and data integration with non-imaging-based genome-wide genetic screens.

(B) Secondary, high-resolution OPS.

(C) New regulators of STING trafficking identified by OPS. Created with BioRender.com.

analyses of image-based features derived from the OPS. This identified clusters of genes that similarly affected STING trafficking, including some that had been previously validated, underscoring the robustness of their approach. They further validated the top 262 genes through a second OPS with even higher content (Figure 1B); using two cell lines, two time points, and 11 imaging channels to track proteins relevant to STING signaling (STING, NF-κB p65, p62), STING trafficking compartments (Calnexin in the ER, GM130 in the Golgi, EEA1 in endosomes), STING degradation pathways (HGS, ATP6V1D), and overall cell state (DAPI/nuclei, actin, γH2AX).

Through their comprehensive analysis, the team achieved a balanced weighting of orthogonal feature groups, leading to the identification of many novel regulators of STING pathway control (Figure 1C). This included C19orf25 and USE1. C19orf25, a gene of previously unclear function, was found to co-cluster with USE1, a well-characterized ER membrane protein involved in Golgi-ER retrograde transport. C19orf25 KO or USE1 KO localized STING to the Golgi and autophagosomes, suggesting their involvement in STING retrograde transportation from Golgi to ER, similar to COPA. KO of them also led to increased STING activation, as evidenced by elevated levels of STING, phosphorylated STING (pSTING), and phosphorylated TBK1. Another new finding was the role of the HOPS complex, which is involved in membrane fusion within the endolysosomal compartment and between autophagosomes and lysosomes. They found that when VPS11 and VPS33A, subunits of the HOPS complex, were knocked out, there was reduced STING degradation, elevated phosphorylation of STING, and impeded autophagic flux, which lead to increased expression of IFN-β and IL-6. The GARP and RIC1-RGP1 complexes were also found to regulate STING. The RIC1-RGP1 complex regulates GDP-GTP exchange of RAB6, involved in the secretory pathway. The GARP complex is involved in recycling between endosomes and the Golgi. They found that KO of RIC1 or VPS52 led to decreased STING degradation and prolonged its presence in the Golgi, increasing pSTING levels.

This study significantly advances our understanding of the dynamic regulation of STING by uncovering the roles of various genetic components involved in its control at different points in its trafficking through the cell, which are tied to its functional life cycle. This has important implications for our understanding of this vital immune process and its role in health and disease. For example, genetic mutations of the HOPS complex, the GARP complex, and the RIC1-RGP1 complex are associated with human disease but they were not linked to STING regulation until this study. The study's insights can also guide drugging of the cGAS-STING pathway. For instance, pharmacological agents that inhibit or enhance STING Golgi exit and degradation could potentially be used to either amplify the immune response in cases such as cancer immunotherapy or attenuate it in autoimmune diseases.

Beyond the relevance to our understanding of STING biology, the study represents the likely future of functional genomics and systems biology. CRISPR screens are often decried as low-information "fishing expeditions" (especially by grant reviewers), but when reading Gentili et al., it is clear this view is outdated. OPS,[8] along with other high-dimensional functional genomics approaches, like Perturb-seq[5] and Perturb-map,[10] demonstrate how deep biological insights into a

pathway or process can be gained from pooled CRISPR screens. This can include determining how different genes affect protein co-localization within a cell (key to understanding pathway control), as done here, or even how specific genes influence cell interactions within a tissue or tumor. Gentili et al. is a model example of the path ahead for systems biology with new technologies, like OPS, being used to deconvolute the high complexity of cellular biology and greatly advancing our understanding of high-component processes, like innate immunity.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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