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Elucidating the Mechanism of Action of STING Degradation

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INTRODUCTION

The cyclic GMP-AMP synthase-stimulator of interferon genes (cGAS-STING) pathway (***Fig. 1A***) plays an important role in innate immune sensing of DNA [1]. Localized at the endoplasmic reticulum (ER), STING is activated by 2',3'-cyclic GMP-AMP (cGAMP), which is produced by the DNA sensor cGAS. STING is then trafficked to the Golgi, where a phosphorylation cascade involving the TANK-binding kinase 1 (TBK1) and interferon regulatory factor 3 (IRF3) is triggered, resulting in interferon-β (IFNβ) expression [1]. STING is an attractive target for therapeutic applications due to its involvement in immunopathogenic diseases [2].

CONCEPT AND AIM

Novartis identified a new compound, which depletes STING in THP1 monocytic cells (***Fig. 1A***). To investigate the mode of action of this compound, a pooled CRISPR-Cas9 knockout screen was conducted (***Fig. 1B***), which identified many gene hits. To investigate the role of selected hits in compound-mediated STING degradation, four project milestones were defined: (1) Review of screening data and hit selection, (2) engineering CRISPR knockout cell lines, (3) validation of the gene knockouts on DNA and protein level, (4) performing functional validations.

RESULTS

Gene hits *A*, *B*, *C*, *D*, *E*, and *F* were selected for in-depth validation. Each gene was knocked out in monocytic THP1-Dual Cas9-expressing cells using CRISPR technology and four T. spiezzo single-guide RNAs (4sg), targeting the same gene. Tracking Indels by DEcomposition (TIDE) and western blot analysis confirmed gene *A*, *B*, *C* and *D* knockout with shares of 35-85% frameshift mutations at selected sgRNA target sites and/or no detectable protein expression in the cell pool (***Fig. 1C/D***).

Knockout cell lines were subjected to functional validation via a STING flow cytometry assay, where cells were treated with the compound, fixed and stained using an anti-STING-GFP antibody. As expected from the CRISPR screen, gene *A*, *B*, and *C* knockout cells revealed an increased STING abundance in presence of the compound compared to the control (CTRL sg) (***Fig. 1E***). Moreover, STING subcellular localization was studied in the knockout cells via immunofluorescence imaging. Results showed STING granules and particles, manly around strongly stained ER regions, which were resolved in presence of the compound (***Fig. 1F***).

In addition, a reporter gene assay was used to monitor the cGAS-STING pathway activation (***Fig. 1G/H***). Surprisingly, a strong compound-dependent inhibition of the cGAS-STING pathway was observed in gene *B* knockout cells (***Fig. 1H***). Furthermore, to test the impact of the gene knockouts on the ER integrity, a xenobiotic ER perturbation assay was conducted by measuring the cell viability upon treatment with a cotransin analogue that blocks ER protein biosynthesis, or tunicamycin, a known ER stressor (***Fig. 1I/J***). Interestingly, gene *B* knockout cells reacted less sensitive to both treatments.

CONCLUSION AND OUTLOOK

This project aimed to validate six genes potentially involved in compound-mediated STING degradation. From the selected hits, genes *A*, *C*, and especially *B* showed promising results by partially rescuing the compound's mode of action. The study also found that gene *B* knockout cells are less sensitive to xenobiotic treatments, indicating that such compounds may not be able to efficiently penetrate these cells. To test this hypothesis further, a small-scale compound screen is planned. This project also highlights the importance of gene-by-gene validation after a CRISPR screen, as some hits might be false-positive. Notably, the THP1-Dual cell line was used for the validation of the candidates in this project due to the convenience of an integrated reporter gene. However, it is important to confirm the results in the THP1 cell line that was originally used in the CRISPR screen. Promising hits identified in this study can then be further investigated for their involvement in compound-mediated STING degradation.

REFERENCES

[1] Motwani, et. al., 2019. DNA sensing by the cGAS–STING pathway in health and disease.
[2] Saldanha, R.G., et. al., 2018. A mutation outside the dimerization domain causing atypical STING-associated vasculopathy with onset in infancy.

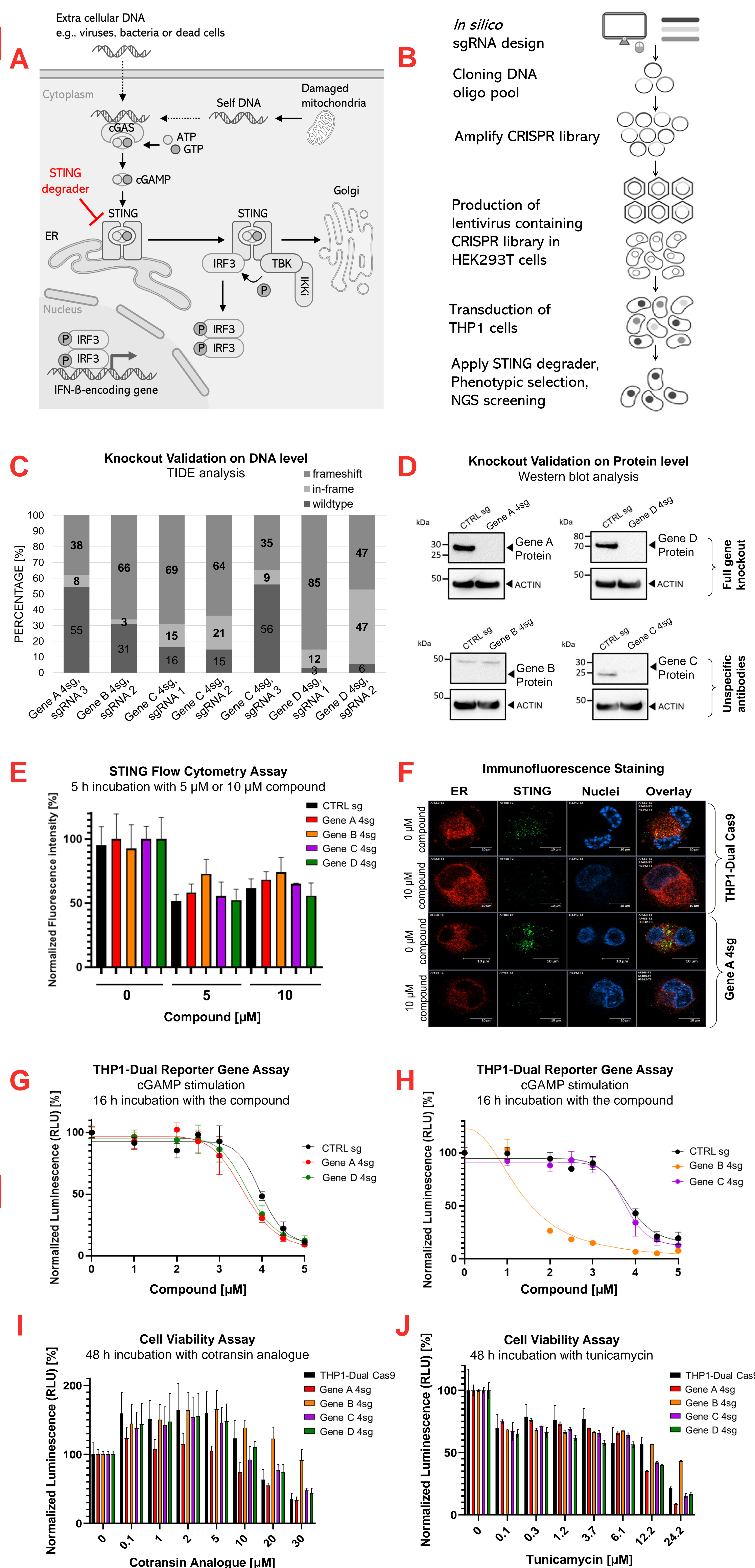

Fig. 1 (**A**) cGAS-STING DNA-sensing pathway. (**B**) Pooled CRISPR screen workflow. (**C**) TIDE analysis of selected sgRNA target sites. (**D**) Western blot of selected knockout cell lines. (**E**) STING flow cytometry assay. (**F**) Confocal microscopy images (63x) of STING, ER, and nucleus stained THP1-Dual Cas9 and Gene *A* knockout cells in absence or presence of 10 µM compound. (**G/H**) THP1-Dual reporter gene assay. (**I/J**) Cell viability assay upon (**I**) cotransin analogue or (**J**) tunicamycin treatment. Error bars represent standard deviation. *Abbreviation: CTRL: control (non-targeting sgRNA transduced THP1-Dual Cas9), 4sg: four T. spiezzo sgRNAs, P: Passage, FACS: Fluorescence-activated cell sorting.*

NOVARTIS