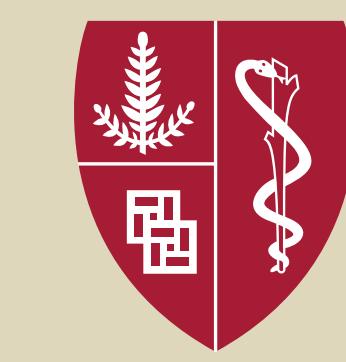


SMASh 2.0: degron optimization for drug-dependent protein degradation

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Background

Small molecule-assisted shutoff (SMASh) is a single-component technique for selective depletion of specific proteins in which proteins are fused to HCV (hepatitis C virus) protease followed by a degron. In presence of protease inhibitor, such as asunaprevir (ASV), the degron removal is blocked resulting in the degradation of new copies of the protein. As such, SMASh allows drug-dependent control over levels of various proteins in multiple mammalian cell types and in yeast.

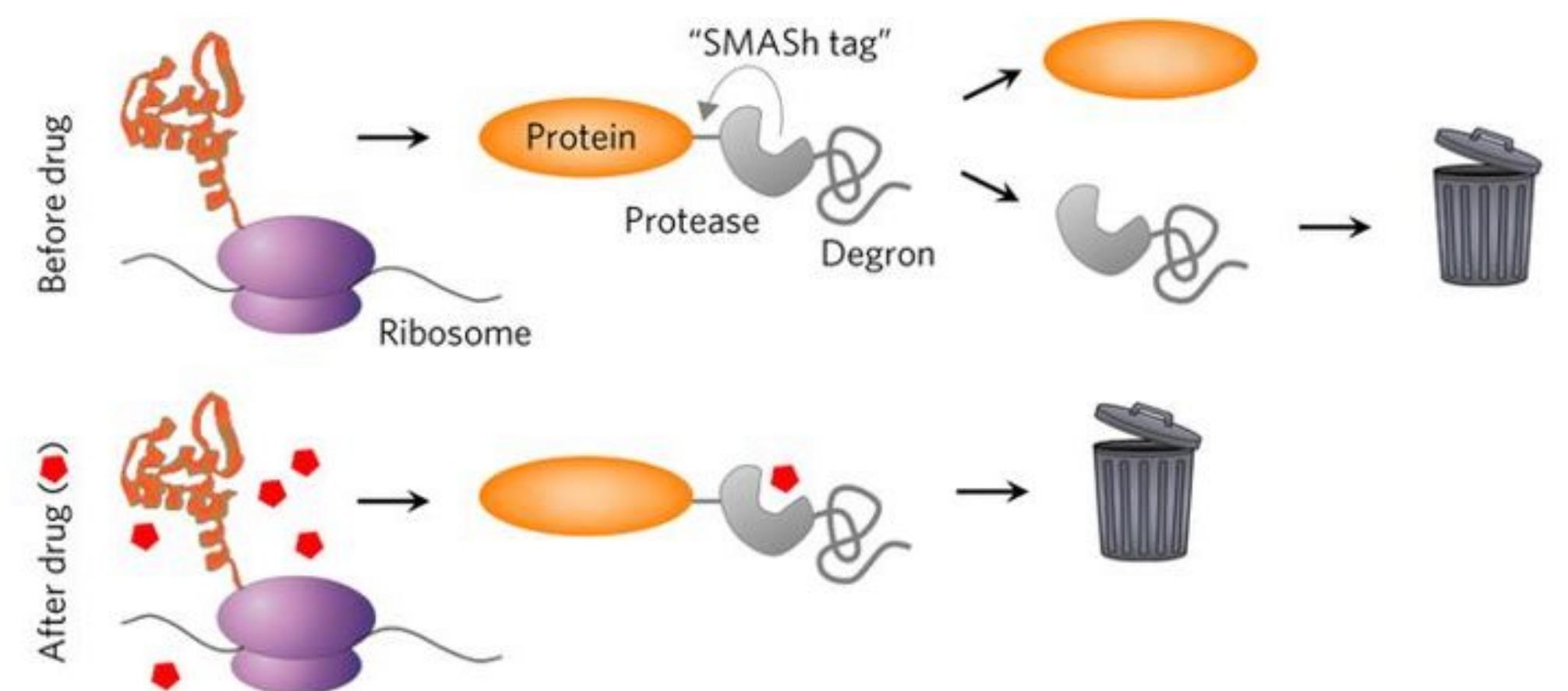


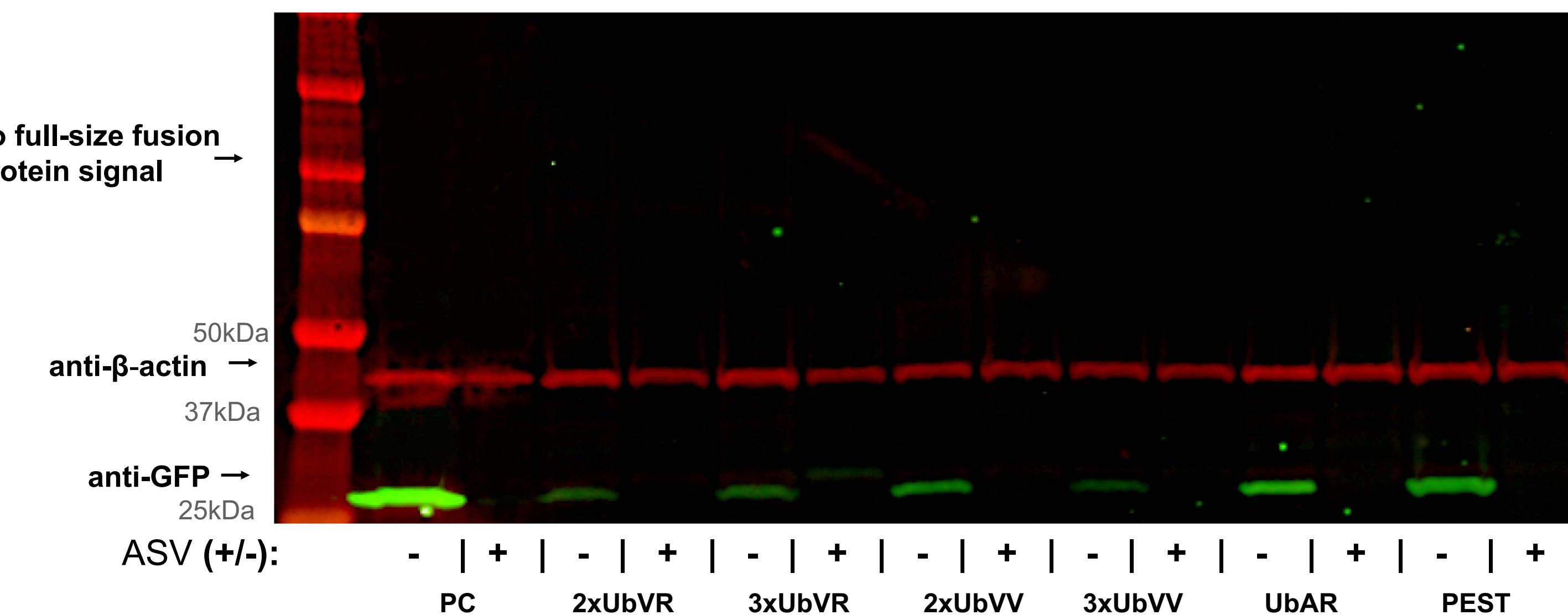
Figure from Nature Chemical Biology: doi:10.1038/nchembio.1869

Objectives

- Previously engineered SMASh tags use NS4A as a degron sequence. NS4A is a nonstructural protein found in HCV that acts as an efficient degron in mammalian cells. Because of viral origin of the peptide, its presence in live human cells may result in unexpected immune response limiting the prospective use of SMASh for in vivo application.
- Our aim for this project was to find and test alternative degron sequences that would allow efficient protein degradation with little immunogenicity.

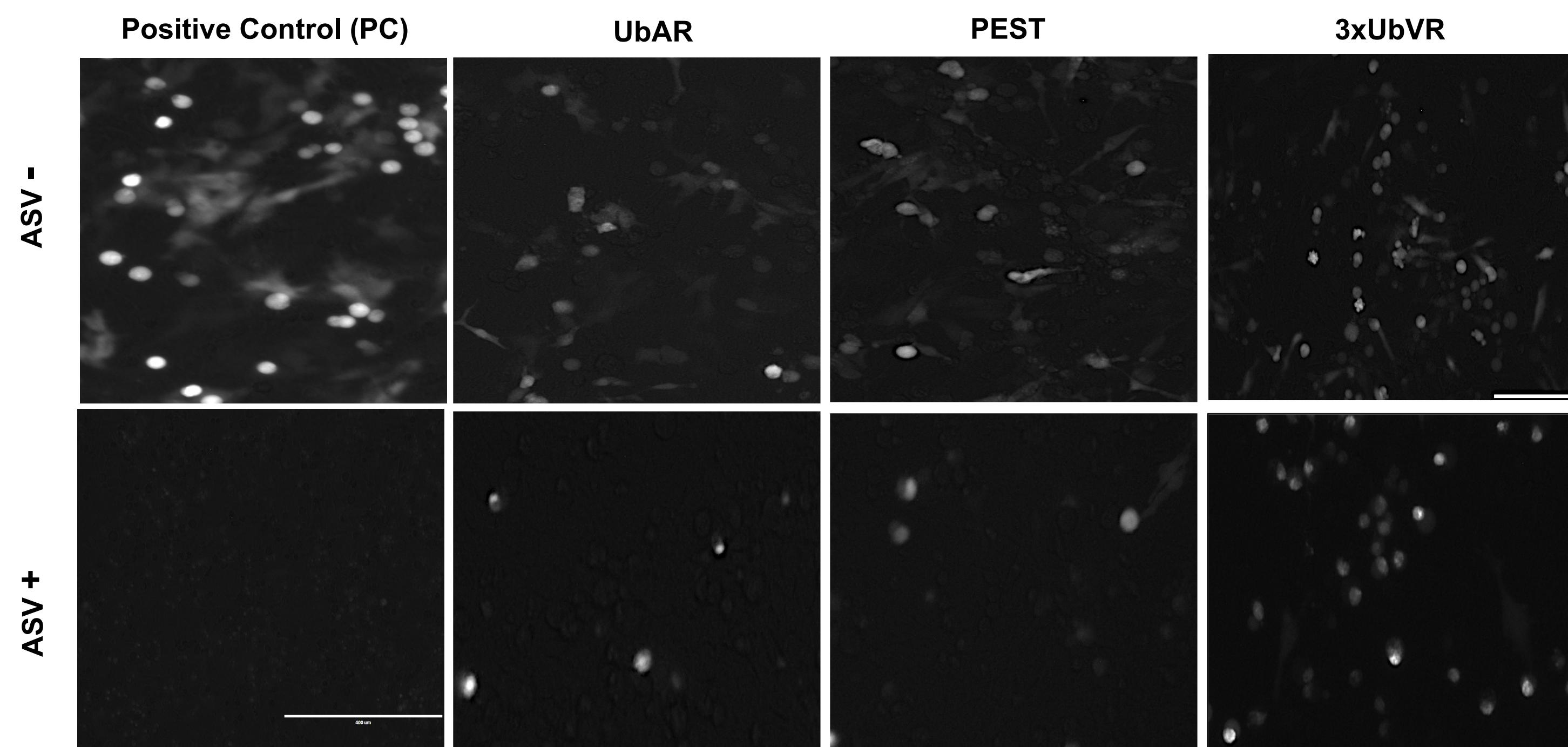
Results

We tested 6 degron sequences from *A modular degron library for synthetic circuits in mammalian cells* (Chassain et al., 2019): 2xUbVR, 3xUbVR, 2xUbVV, 3xUbVV, UbAR, PEST. Ubiquitin(Ub)-proteosome pathway degrades the majority of intracellular proteins in mammalian cells, thus using Ub-containing degron should minimize the immunogenicity of SMASh2.0 tag. Immunoblotting results had confirmed the destabilizing effects of 5 out of 6 sequences. In the absence of ASV, a 30-kDa YFP fragment was released as expected. In contrast, in the presence of ASV, no full-length fusion protein (YFP-NS3pro-degron) was detected.



The only sequence that had an observable signal in presence of ASV was 3xUbVR. 2xUbVR, 3xUbVR, and 3xUbVV have a very weak signal in the absence of ASV. We hypothesize that these results could be due to the use of the enhanced promoter. High levels of expression of the fusion protein could result in protein degradation system being overloaded, and, therefore, reduce the observed signal as proteins are not being degraded fast enough before the cell dies.

We further used fluorescent microscopy to test this hypothesis. In absence of ASV, all cells had shown fluorescence as expected. After ASV treatment, live cells with new constructs had little to no fluorescence. We observed fluorescence only in dead cells, which confirmed our hypothesis.



Methods

DNA constructs

Initial DNA sequences of degrons and primers were obtained from IDT. Plasmids encoding YFP and NS3 protease were fused to degron sequences by standard techniques including PCR, double digestion and In-Fusion cloning to create new SMASh variants. All fragments were sequenced to confirm successful construction.

Cell culture and transfection

HeLa cells were used for transfection. Cells were cultured at 37 °C in 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, glutamine, penicillin and streptomycin; transfected using Lipofectamine 3000 in Opti-MEM according to the recommended protocol.

Immunoblotting

After 48h, cell were lysed using hot SDS buffer with 2-mercaptoethanol, sonicated and heated to 90°C. Cell lysates were loaded on SDS-page gel, transferred to nitrocellulose membrane and probed with primary (Mouse anti-β-actin; Rabbit anti-GFP) and fluorescent secondary antibodies(Goat-anti-mouse; Goat-anti-rabbit). Imaged using OdysseyCRx.

Conclusions

- Due to the simplicity of the design, SMASh technique can be useful for a variety of problems in biotechnology where the control over production of a specific protein is desired. The applications range from studies of a gene or protein function to engineering oncolytic viruses for cancer treatment.
- Use of new SMASh variants with UbAR, PEST, or 2xUbVV degron sequences can be a non-toxic alternative for future in vivo biomedical research.

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