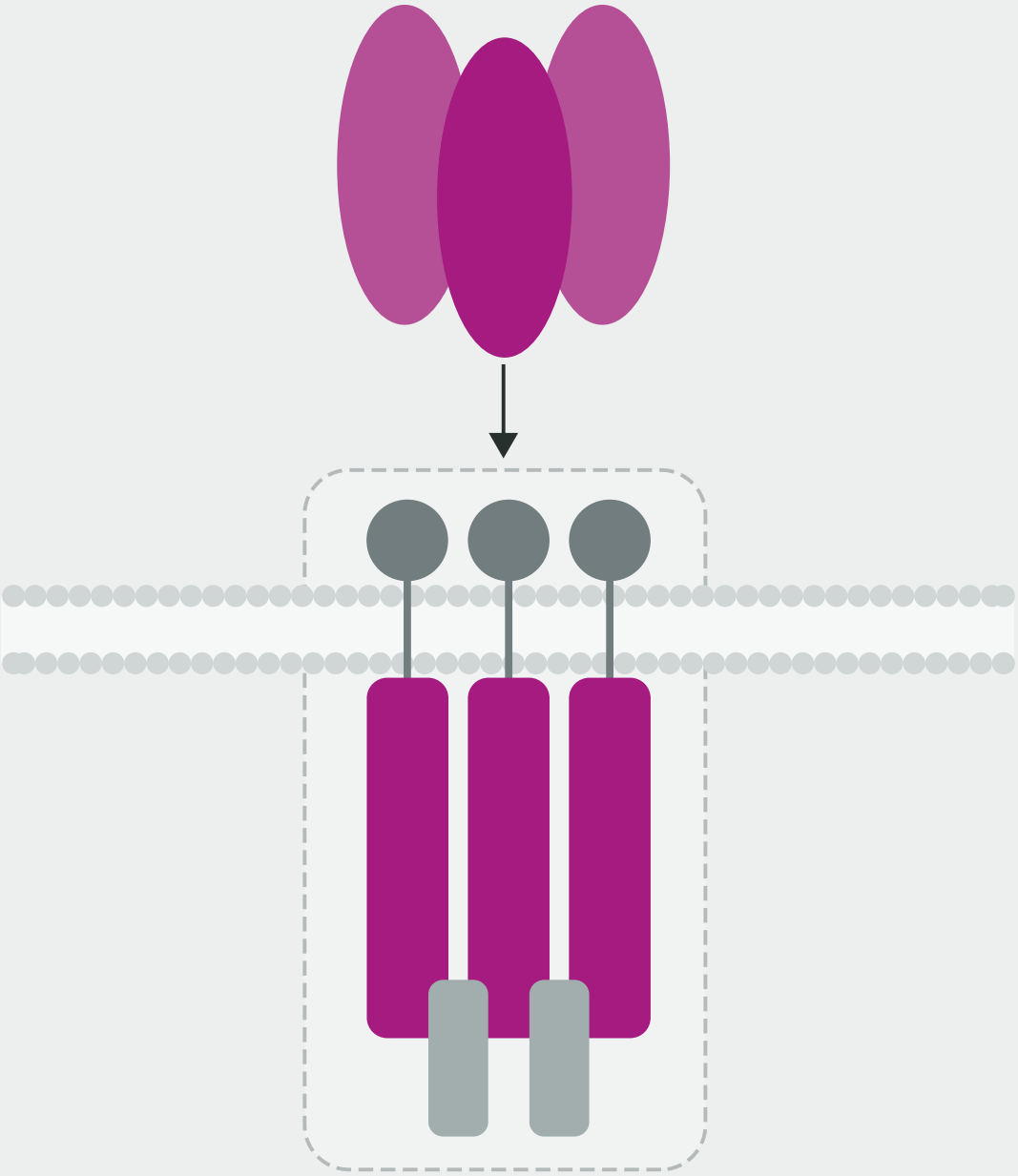
abcam

Application note

Knock-out cell lines and their use in signaling pathways



Contents

Summary

Knock-out (KO) cell lines are powerful tools to study disease mechanisms, such as the effect of gene disruption on downstream pathways. In this application note, we highlight the effectiveness of Abcam KO cell lines that can be used to investigate signaling pathways, in this case, the TRAIL pathway.

TRAIL (TNF-related apoptosis-inducing ligand) is a protein cytokine that causes apoptosis through binding to several death receptors. These receptors have gained substantial interest as cancer cells are significantly more sensitive to TRAIL-induced apoptosis than normal cells.

We used a KO cell line of a TRAIL death receptor, DR5, in HeLa cells (ab264922) to demonstrate that KO attenuates the downstream apoptotic pathway initiated by TRAIL. Upon activation of the pathway, the signal is propagated through a series of effector caspases, including caspase 3. Therefore, we included a small-molecule caspase 3 inhibitor, Q-DEVD-OPh, as a positive control.

Demonstrating the advantage of a complete genetic KO, the DR5 KO line had a greater effect on inhibiting the downstream signaling pathway than Q-DEVD-OPh at the concentrations used.

Introduction

The evolution of CRISPR (clustered regularly interspaced short palindromic repeats)-Cas (CRISPR-associated) 9 technology has had a huge impact on biological research[1,2,3,4]. In addition to the capacity to develop disease models, CRISPR-Cas9 has revolutionized reagent validation and studies into disease mechanisms.

CRISPR development has cut pipeline attrition during drug discovery, aiding target identification, high-throughput screening, hit validation, and lead optimization[5]. It has also proved to be a critical tool for disease researchers, enabling evaluation of downstream signaling effects to explore the basis of many disorders[6]. Additionally, CRISPR-Cas9 can be used to create KO cell lines, which play an integral role in both drug discovery and disease research.

In this application note, we highlight how KO cell lines can be used to investigate signaling pathways, in this case, the TRAIL pathway. We also included the caspase 3 inhibitor Q-DEVD-OPh as a positive control. It is worth noting that, while small-molecule inhibitors can block the activity of the protein, the protein is still physically present, allowing it to act as a scaffold for functions such as protein-protein interactions[7]. However, small-molecule inhibitors can be used synergistically with KO cells, forming an exciting approach for drug discovery[8].

TRAIL (TNF-related apoptosis-inducing ligand) is a protein cytokine that causes apoptosis through binding to several death receptors, including DR4 and DR5[9,10]. This binding activates the extrinsic apoptotic pathway to induce apoptosis through a cascade of effector proteins, including caspase-3 and caspase-7[11].

Poly (ADP-ribose) polymerase (PARP1) binds to DNA lesions as part of the cell's DNA repair mechanism[12]. However, during the extrinsic apoptosis pathway, PARP1 is cleaved by caspase 3 and caspase 7, resulting in loss of activity and suppression of DNA repair[12]. In recent years, TRAIL receptors have gained substantial interest as cancer cells are significantly more sensitive to TRAIL-induced apoptosis than normal cells[13].

Here, we demonstrate that the loss of death receptor 5 (DR5) by CRISPR gene KO, blocks the downstream processing associated with TRAIL signaling, and has a greater effect than Q-DEVD-OPh at the concentrations used.

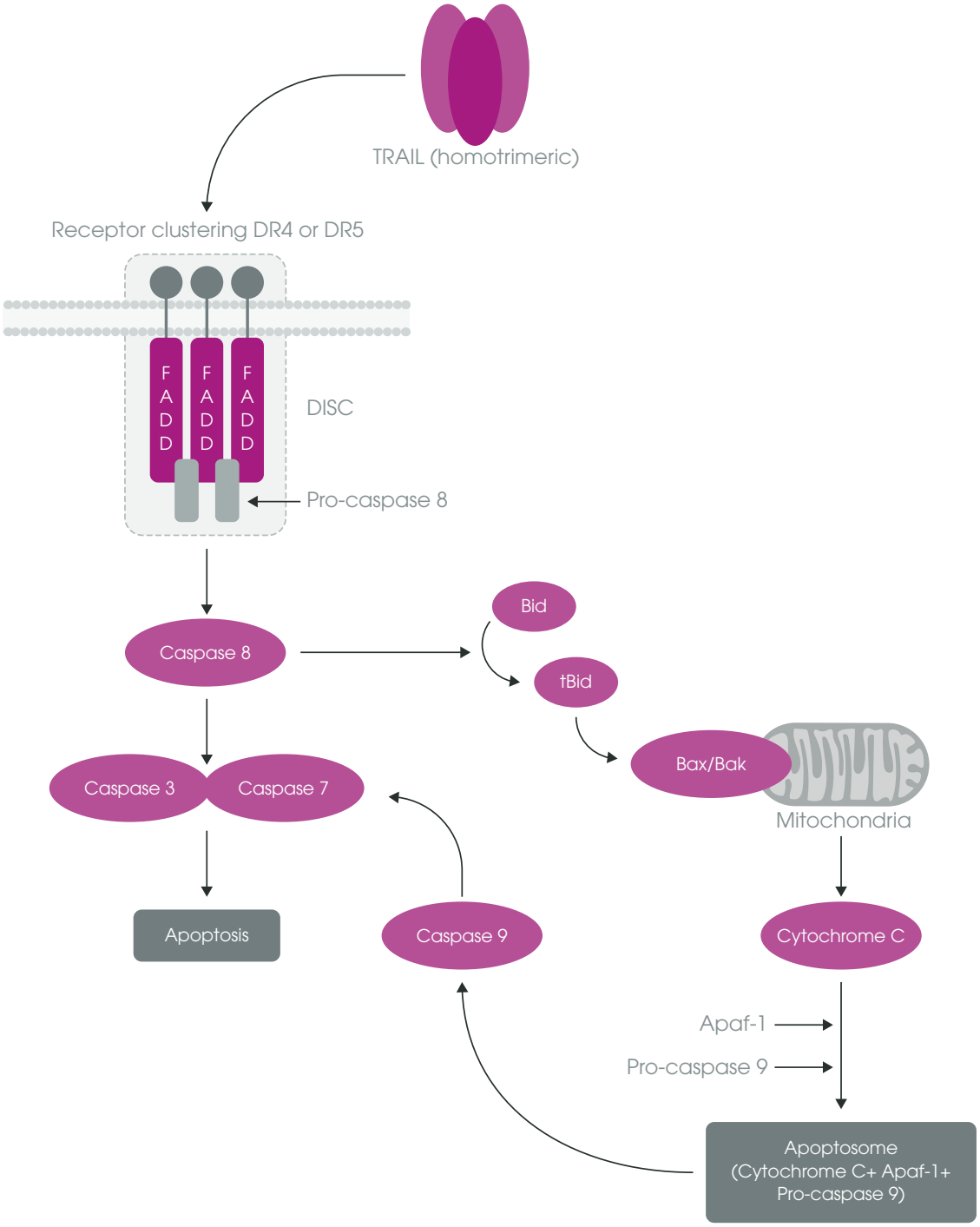


Figure 1. The TRAIL-induced apoptotic pathway

Materials and methods

- Human TNFRSF10B (DR5) knock-out HeLa cells (ab264922)
- Human wild type HeLa cells (ab255450)
- Recombinant human TRAIL protein (ab9960)
- Caspase 3 inhibitor Q-DEVD-OPh (ab142037)
- Lysis buffer (ab152163)

TNFRSF10B (DR5) knock-out

TNFRSF10B (DR5) was knocked out in human HeLa cells by transfection with three components: a plasmid expressing Cas9, a plasmid expressing the gRNA, and a plasmid expressing the linear DNA. The plasmid containing the linear DNA comprises a selection cassette of 1 kb that confers puromycin resistance and includes stop codons in the three reading frames. Insertion into the targeted site is confirmed by PCR using specific primers that encompass the targeted site. The insertion induces a stop codon and the bands with the insertion are submitted to sequencing. The KO was validated via western blot.

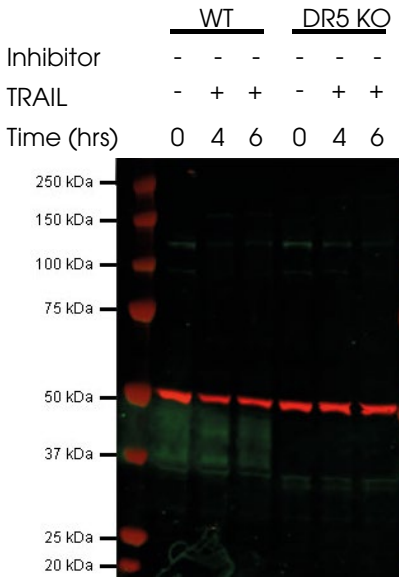


Figure 2: western blot showing expression of DR5 at 0, 4, and 6 h in wild type and knock-out cells

Cell culture

Human TNFRSF10B (DR5) knock-out HeLa cells (ab264922) and human wild type HeLa cells (ab255450) were seeded into 15 cm dishes with DMEM + 10% FBS and incubated overnight at 37°C. The cells were subsequently treated with recombinant human TRAIL protein at 100 ng/mL (ab9960) either in the presence or absence of caspase 3 inhibitor Q-DEVD-OPh at 200 µM (ab142037). Both cell lines without TRAIL treatment were also included as negative controls. At various time points, the cells were then harvested using lysis buffer (ab152163) at 4°C.

Western blot

Protein content was quantified using a BCA assay and the lysates were normalized before western blotting. 20 µg lysate was resolved using NuPAGE 4-12% Bis-Tris gels (Life Technologies Ltd, NP0336BOX). Proteins were transferred to nitrocellulose membranes (Life Technologies Ltd, LC2000) using the X-cell SureLock™ mini cell system, by applying a constant voltage at 30 V for 1.5 h. The membranes were then blocked for 1 h with 5% milk, TBST (Tris-buffered saline with 0.1% Tween-20 (v/v)).

The membranes were then probed with the appropriate primary antibody (at assay dependent concentrations) and loading control antibody before incubation overnight at 4°C with agitation. The following day the membranes were washed three times with TBST, before incubation for 1 h with IRDye secondary antibodies (LI-COR) diluted 1/20,000 in 5% milk, TBST. The membranes were then washed a further four times for 10 min with TBST and imaged using a LI-COR Odyssey imaging system.

Target	abID	Dilution factor
DR5	ab199357	1/1000
Caspase 3	ab32351	1/5000
Caspase 7	ab256469	1/1000
PARP1	ab32138	1/1000

Results

Caspase 3

When TRAIL is added to wild type (WT) cells, the 17 kDa band shows that caspase 3 is present at 0 h but levels decrease at 4 and 6 h. This shows that the addition of TRAIL has activated the cascade and facilitated the processing of caspase 3. In contrast, the amount of caspase 3 in the DR5 KO cells after treatment with TRAIL does not change over time, showing the lack of signal transduction. The processing of caspase 3 is also blocked by the inhibitor Q-DEVD-OPh (Q) in WT cells, although to a slightly lesser degree.

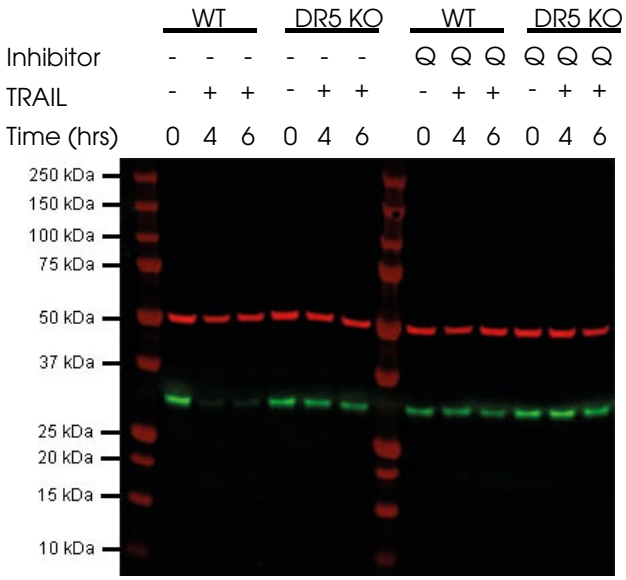


Figure 3: western blot showing expression of caspase 3 at 0, 4, and 6 h in wild type and knock-out cells, with and without Q-DEVD-Oph.

Caspase 7

Caspase 7, another effector caspase in the pathway, is cleaved in response to upstream signals. Upon treatment of WT cells with TRAIL, cleaved caspase 7 (~18 kDa) is not present at 0 h but presents a strong signal at 4 h, with a slight decrease by 6 h. This shows the correct processing of caspase 7 in the signaling pathway. However, when DR5 KO cells are treated with TRAIL, cleaved caspase 7 is not present at any time point, indicating that the absence of DR5 has blocked the signaling cascade.

In WT cells treated with both TRAIL and inhibitor Q, processing of caspase 7 was only partially inhibited, again highlighting the advantage of a KO cell line.

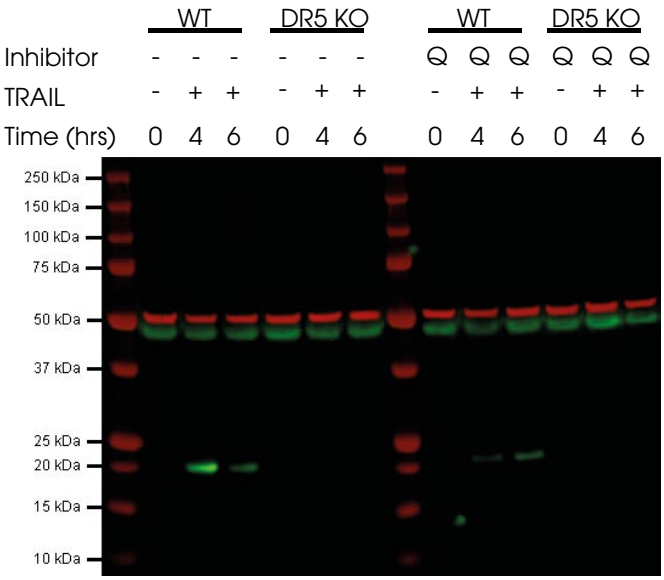


Figure 4: western blot showing expression of cleaved caspase 7 at 0, 4, and 6 h in wild type and knock-out cells, with and without Q-DEVD-Oph. The band at 48 kDa is a non-specific, unidentifiable protein.

PARP

Full-length PARP1 (~125 kDa) is cleaved by caspases 3 and 7, producing a 24 kDa fragment. This fragment is present at 4 h and 6 h after treatment with TRAIL in WT cells. In contrast, this cleaved fragment is not detected in the DR5 KO cells and is partially diminished upon treatment with inhibitor Q.

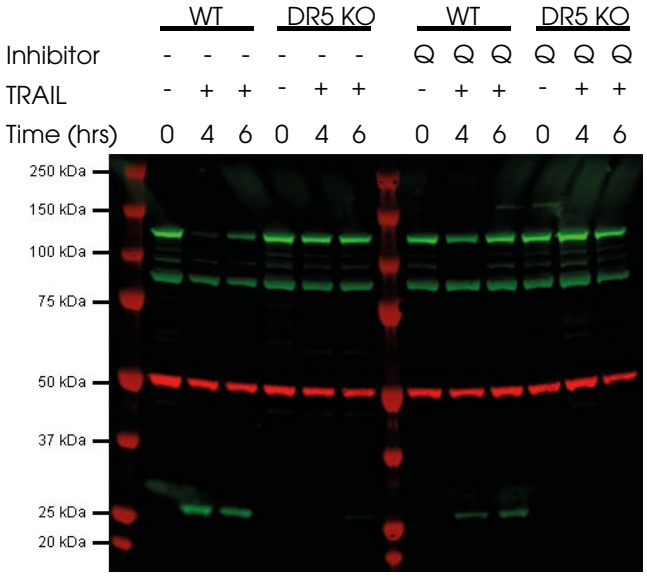


Figure 5: western blot showing expression of PARP1 at 0, 4, and 6 h in wild type and knock-out cells, with and without Q-DEVD-Oph.

Conclusion

Abcam KO cell lines are powerful tools to study disease mechanisms, such as the effect of gene disruption on downstream pathways, and can play a critical role in identifying future generations of pharmaceutical targets.

This application note demonstrates that the KO of DR5 in HeLa cells attenuates the downstream apoptotic pathway initiated by TRAIL. At the concentrations used, the caspase 3 inhibitor Q-DEVD-OPh had a more limited effect than the KO, demonstrating the power of KO cell lines. This emphasizes how the reliability and potency of a genetically defined KO offers numerous advantages over transient knock-down approaches.

An important point to note is that the difference between complete removal of a protein and chemically inhibiting the enzymatic activity of a protein can change the outcome of an experiment. It may be preferable to use both of these orthogonal approaches to validate one another.

However, it is important to ensure that your KO cell line is extensively validated. To avoid unnecessary delays in your workflow, ready-made cell lines or custom gene-edits can be used. Abcam provides a unique portfolio of KO cell lines to ensure researchers can access the high-quality cell lines they need to study the biological function of their gene of interest or rapidly progress drug development.

References

1. Lino, C., Harper, J., Carney, J. & Timlin, J. Delivering CRISPR: a review of the challenges and approaches. *Drug Delivery* **25**, 1234-1257 (2018).

2. Ishino, Y., Krupovic, M. & Forterre, P. History of CRISPR-Cas from Encounter with a Mysterious Repeated Sequence to Genome Editing Technology. *Journal of Bacteriology* **200**, (2018).

3. Zhang, F., Wen, Y. & Guo, X. CRISPR/Cas9 for genome editing: progress, implications and challenges. *Human Molecular Genetics* **23**, R40-R46 (2014).

4. Torres-Ruiz, R. & Rodriguez-Perales, S. CRISPR-Cas9 technology: applications and human disease modelling. *Briefings in Functional Genomics* **16**, 4-12 (2016).

5. CRISPR cell engineering to speed the drug pipeline | Abcam. Abcam.com (2021). at www.abcam.com/reagents/crispr-cell-engineering-to-speed-the-drug-pipeline

6. CRISPR-Cas9 technology: cell knock-out generation | Abcam. Abcam.com (2021). at www.abcam.com/reagents/generating-cell-knockouts-with-crispr-cas9-technology

7. Sangree, A. Not all loss-of-function approaches are created equal: differential cellular responses to small molecule inhibition, acute CRISPR perturbation, and chronic genetic knockout. *Nature Research Bioengineering Community* (2021). at https://bioengineeringcommunity.nature.com/posts/60777-not-all-loss-of-function-approaches-are-created-equal-differential-cellular-responses-to-small-molecule-inhibition-acute-crispr-perturbation-and-chronic-genetic-knockout

8. Szlachta, K. *et al.* CRISPR knockout screening identifies combinatorial drug targets in pancreatic cancer and models cellular drug response. *Nature Communications* **9**, (2018).

9. Wiley, S. *et al.* Identification and characterization of a new member of the TNF family that induces apoptosis. *Immunity* **3**, 673-682 (1995).

10. Walczak, H. TRAIL-R2: a novel apoptosis-mediating receptor for TRAIL. T*he EMBO Journal* **16**, 5386-5397 (1997).

11. Amarante-Mendes, G. & Griffith, T. Therapeutic applications of TRAIL receptor agonists in cancer and beyond. *Pharmacology & Therapeutics* **155**, 117-131 (2015).

12. Mashimo, M. *et al.* The 89-kDa PARP1 cleavage fragment serves as a cytoplasmic PAR carrier to induce AIF-mediated apoptosis. *Journal of Biological Chemistry* jbc. RA120.014479 (2020). doi:10.1074/jbc.ra120.014479

13. Yuan, X. *et al.* Developing TRAIL/TRAIL death receptor-based cancer therapies. *Cancer and Metastasis Reviews* **37**, 733-748 (2018).