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Investigation of the Neddylation Pathway in Triple Negative Breast Cancer

Liam Patrick Alford

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Biology Education Centre and Department of Immunology, Genetics and Pathology, Uppsala University
Supervisors: Yumeng Mao and Irineos Papakyriacou
External opponent: Oscar Badillo

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Abstract

Triple negative breast cancers (TNBCs) are a subtype of breast cancer that lack three specific membrane proteins and are thus harder to treat with both classical approaches and immune checkpoint blockade therapy. NEDD8 is a protein that plays an important role in post translational protein modification and its substrates often impact cell cycle progression in a substantial manner. It has also been presented as a resistance gene to anti-PD-1 therapy in TNBC. Presently, there are two small molecular inhibitors for the neddylation pathway (pevonedistat and TAS4464), however there are many unknowns regarding their efficacy, safety, and mode of action. Here, it has been demonstrated that pevonedistat resistance leads to significantly lower secretion of granzyme B in a human TNBC model *in vitro*. Based on these results, a murine breast cancer model was generated to further test the effects of resistance *in vivo*. Moreover, other components of the neddylation pathway were investigated. In particular, the regulatory subunit of the NEDD8-activating enzyme, NAE1, and conversely the proposed target of pevonedistat binding. Results showed that not only is NAE1 essential in the cell line used, but they also further validated the actual lack of specificity of pevonedistat cell killing. Since IRAK3 has been proposed as a novel checkpoint in myeloid cells it was worthwhile investigating the effect NEDD8 knockout tumors would have on IRAK3 knockout mice, with or without immune checkpoint blockade therapy. Results showed slight differences between the two conditions, however, much remains to be done in this regard.

Introduction

Breast cancer is the most common cancer occuring in women worldwide (Boyle, 2006; Jemal et al., 2011). Certain subtypes of breast cancer are harder to treat (Maqbool et al., 2022), hence there is a need to explore different intracellular pathways as targets for novel treatment approaches. Here, the influence of the neddylation pathway on therapy resistance in triple negative breast cancer will be explored.

Triple negative breast cancer

Based on specific cellular markers that represent objectives for targeted therapeutic approaches, breast cancers are categorized into three general groups (Engebraaten et al., 2013):

1. estrogen receptor (ER) or progesterone receptor (PR) positive
2. human epidermal growth factor receptor 2 (HER2) positive, with or without ER and PR positivity
3. triple-negative breast cancer (TNBC), which is defined by absence of ER/PR expression and HER2 amplification

Triple-negative breast cancer encompasses 10-20% of invasive breast cancer that involves more than one molecular subtype. The major elements of TNBC are basal-like tumors, normal breast-like tumors, and claudin-low molecular subtypes. TNBC is a very aggressive type of cancer, it presents itself very heterogeneously regarding pathology, clinical presentation, as well as response to various treatment options. Triple negative breast cancer has some general characteristics that are worth noting (Carey et al., 2006; Perou et al., 2000; Rakha et al., 2006):

a) it is frequently seen in women younger than 50 years of age
b) it is more frequent in african-american women and black ethnicities more broadly
c) it usually presents as an interval cancer
d) it has high chemosensitivity
e) there is a weak association between tumor size and lymph node metastases

f) TNBCs have a higher chance of brain metastases and recurrence during the first and third year following diagnosis

g) compared with other subtypes of breast cancer, TNBC has a shorter survival time following a first metastatic event

Proteins often mutated in TNBC that are associated with shorter survival as detected by immunohistochemistry are p53, p15, cKIT, cytokeratins 5/6, 14, and 17, cyclin E and, EGFR (Cheang et al., 2008; Nielsen et al., 2004). Current treatment options for TNBC include surgery, whether it be a complete mastectomy or breast-conserving, radiation therapy, chemotherapy, molecular targeted therapy (i.e. VEGF, EGFR inhibitors) and immunotherapeutic approaches (Schneider & Miller, 2005; Stagg & Allard, 2013). Clinical data has shown that patients who receive primary chemotherapy show a response rate ranging from 27 to 60% based on the therapeutic agent used (platinum-based triplets, taxanes and anthracyclines). Unfortunately, a significant response to chemotherapy does not lead to better overall survival outcomes in the long term, coining this as the "triple negative paradox". Immunotherapeutics that are most commonly used for the treatment of TNBC include anti-PD-1 or anti-PD-L1 therapy, and CAR-T cells (P. Kumar & Aggarwal, 2016; Rastelli et al., 2010).

Neddylation pathway

Neddylation is one of the most important post-translational modifications and is a key regulator of protein homeostasis in eukaryotic cells. It involves the conjugation of the ubiquitin-like protein NEDD8 to its substrates, and it encompasses nearly 20% of all ubiquitinated proteins. Although very similar to ubiquitination, neddylation has very specific substrates, some of which are involved in the process of oncogenesis and cancer progression (Foster et al., 2021; L. Zhou et al., 2019).

Neural precursor cell-expressed developmentally downregulated 8 (NEDD8) is a protein coded by the NEDD8 gene. It is a protein that shows a high similarity both in protein structure and sequence to ubiquitin (S. Kumar et al., 1992). Indeed, NEDD8 is involved in the process of conjugation and post-translation modification of several proteins, most notably Cullin-RING ligases (CRLs), a group of proteins that are involved in several important cellular processes, such as contributing to cell cycle progression and cellular survival (L. Zhou et al., 2018; Zou &

Zhang, 2021). During protein neddylation, NEDD8 activates Cullin-RING ligases or non-cullin substrates in a process that involves several key steps (Figure 1) (Watson et al., 2011; L. Zhou et al., 2018):

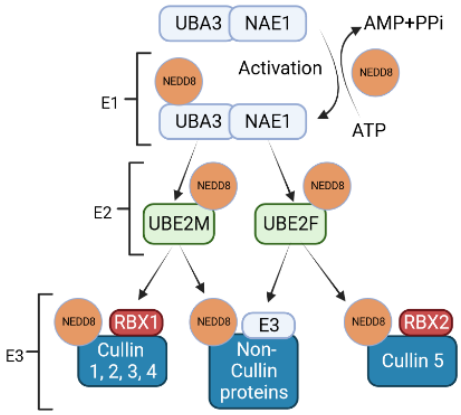

Figure 1: Overview of the Neddylation pathway (created using www.biorender.com)

1) a deneddylating enzyme (NEDP1, CSN, USP21, etc.) hydrolyzes the NEDD8 precursor form at a specific C-terminal glycine residue to form mature NEDD8
2) the mature NEDD8 is first adenylated and activated in an ATP-dependent manner by the action of the NEDD8- activating enzyme E1 (NAE) that is a heterodimer made up by APP-BP1 (NAE1) and UBA3
3) next, an activated NEDD8 is transferred to E2 conjugating enzymes (UBC12, UBE2F)
4) an E3 enzyme transfers NEDD8 onto a substrate (RBX1, RBX2)
5) for CRLs to be activated, they require neddylation of the cullin subunit
6) activated CRLs transfer ubiquitin to a protein substrate to form a polyubiquitin chain which leads to protein degradation via the ubiquitination system
7) neddylation itself is a modification that can be reversed. This is accomplished via the action of several deneddylating enzymes including SENP8, COP9 Signalosome (CSN) etc. (Y. Lu & Yang, 2020).

The role of neddylation in cancer

Several cancer types have an overexpression of key components of the neddylation pathway, namely different cullin proteins. Cullin 1 (Cul1), complexed as CRL1 (SCF) is a prime example; it is involved in the degradation of cell cycle progression inhibitors (e.g. p21 and p27) (Z. Lu & Hunter, 2010). Moreover, β-Trcp has shown to be an indicator of poor prognosis in cancer substrate specific E3 ligases. $SCF^{\beta\text{-}Trcp}$ acts as a promotor of degradation for IκB, which in turn positively affects the NF-κB pathway. This pathway is extensively involved in the development and progression of cancer since NF-κB affects several target genes (TNF-α, Bcl-2, VEGF, IL-6 etc.) and thereby controls cancer cell proliferation, survival, and angiogenesis (Xia et al., 2018). Furthermore, CRL3 complexes have roles in, for example, cytokinesis regulation, responses to oxidative stress, Wnt-β-catenin pathways, while CRL4 complexes play part in nucleotide excision repair and DNA replication. To protect cells from oxidative stress a subunit of BTB (KEAP1) has an interaction with Cul3 which in turn promotes the degradation of NRF2, a transcription factor that gets activated in the presence of reactive oxygen species, and then transactivates genes containing an antioxidant response element. Some proteins that have long been linked to cancer, like p53 and MDM2 have also been established as non-cullin substrates of NEDD8 (Watson et al., 2011).

Pharmacological inhibitors of the neddylation pathway and immune checkpoint blockade

Because the neddylation pathway plays a key role in sustaining cancer proliferation and progression, it has been reported as a target for anti-cancer therapy. Thus, pharmacological inhibitors, such as pevonedistat (MLN4924), have been developed to act against the neddylation pathway. Pevonedistat is an inhibitor of the NAE enzyme that prevents the activation of CRLs in a selective manner thereby changing the ubiquitination and proteasomal degradation of cellular proteins (Figure 2). Literature data showed that inhibition of the neddylation pathway and the resulting inactivation of CRLs leads to cellular processes such as cell cycle arrest, senescence, apoptosis, and autophagy *in vitro* (Y. Lu & Yang, 2020; Torka et al., 2020).

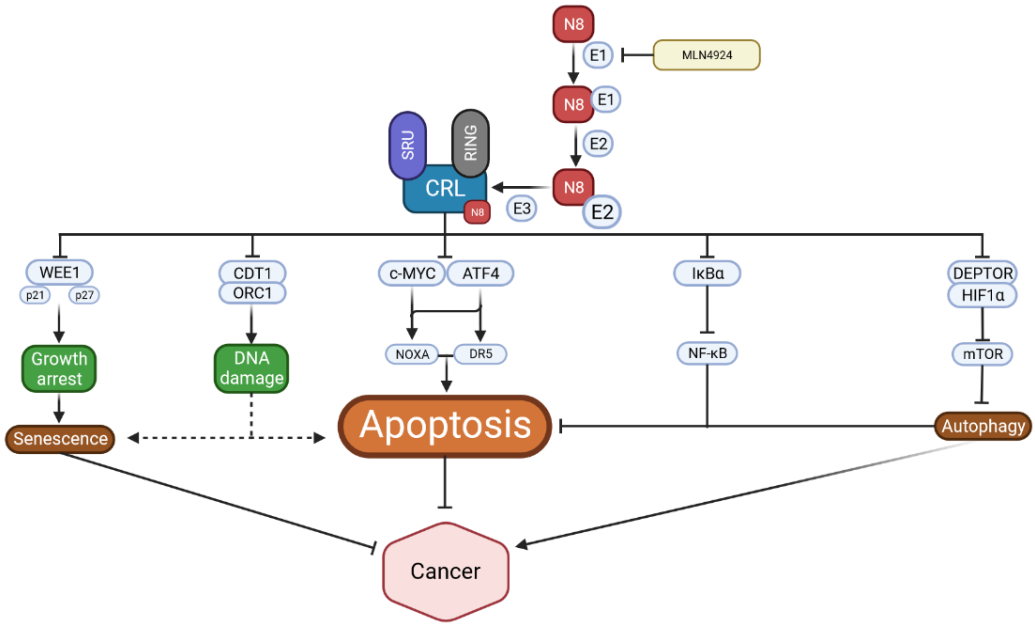

Figure 2: Pevonedistat triggers multiple anti-cancer mechanisms

Pevonedistat has been shown to have a potent effect on several microsatellite instable (MSI) cancer cell lines, including colorectal and endometrial cancer cell lines. Its efficacy was demonstrated both *in vitro* and *in vivo* in xenograft mouse models where inhibition of neddylation lead to reduced growth of tumors even in cells that had a resistance to chemotherapeutics. While the treatment had a significant impact on MSI models, microsatellite stable (MSS) cell lines were not affected. This is due to the fact that pevonedistat leads to the accumulation of unmodified mutant proteins followed by immunogenic cell death in MSI cells, which might imply that neddylation is required for the clearance of mutant proteins. In fact, during cellular stress there occurs a co-conjugation of NEDD8 and ubiquitin that is also successfully inhibited by pevonedistat (McGrail et al., 2020).

The treatment of MSI cell lines with pevonedistat also led to an increased expression of several inflammatory factors which brought about substantial immune cell migration toward the tumor cells (McGrail et al., 2020). Even though pevonedistat gave promising results both *in vitro* and then *in vivo* in mouse models its phase 3 trial was stopped by the manufacturer, who gave the following statement: “The phase 3 PANTHER (Pevonedistat-3001) study did not achieve pre-defined statistical significance for the primary endpoint of event-free survival” (Takeda Pharmaceutical Company Limited). As a result, this failure has now created the need for research

on new therapeutics that could affect the neddylation pathway in a beneficial way in the treatment of cancer.

Immune checkpoints represent a diverse set of receptors such as PD-1 (programmed cell death protein 1), PD-L1 (programmed death-ligand 1), CTLA4, and LAG3 (lymphocyte-activation gene 3) that can act as co-stimulatory or inhibitory signals in the regulation of T-cell activation The general role of PD-1 is in limiting T-cell activity in peripheral tissues at times of infection-induced inflammatory responses. Once activated, T-cells express PD-1 on their surface. Binding of one of the two ligands of PD-1, PD-L1 and PD-L2 leads to inhibition of T-cell activation kinases, thereby inhibiting the function of these immune cells (Pardoll, 2012). Since T-cells have the ability to recognize peptides from every cellular compartment in a selective manner, they have become a major interest for therapeutic manipulation in antitumor immunity. Even though inhibitory signals in the immune response target intracellular signaling pathways, they are usually initiated by membrane-bound receptors or cytokines. Inhibitory receptors and other ligands that affect T-cell function are, however, most commonly overexpressed on cells in the tumor microenvironment (on both cancer cells and non-transformed cells) (Pardoll, 2012). Therapeutic antibodies that have been developed to block immune checkpoints target T-cell receptors and their ligands as opposed to targeting cancer cells directly (Figure 3) (Pardoll, 2012). Furthermore, a combination of pevonedistat and anti-PD-1 had an even larger anti-tumor effect with the efficacy in mice being 6 out of 10 with a complete response whereas each monotherapy had only a 2 in 10 response according to McGrail et al. It has been demonstrated in several studies on multiple cancer cell lines that a higher mutational burden in cells positively affects the response to immunotherapy since there is a larger probability that the cancer cells will express neoantigens that could be recognized by the immune system. Furthermore, inhibition of the neddylation pathway has also led to the upregulated expression of PD-L1 and increased the efficacy of immune checkpoint blockade in glioblastoma cancer cells (Lu & Yang, 2020).

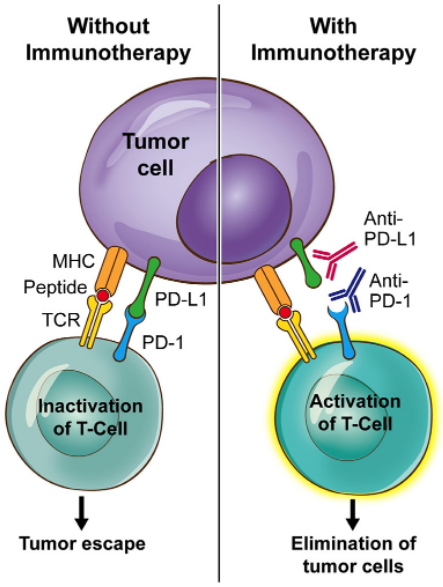

Figure 3: Example of immune checkpoint blockade mode of action (Soularue et al., 2018)

Moreover, research previously conducted in my host research group has shown that NEDD8 acts as a resistance gene to anti-PD-1 immune checkpoint blockade therapy (ICB) (I. Papakyriacou, unpublished data).

IL-1 receptor-associated kinase-3 (IRAK3) has been proposed as an immune checkpoint in myeloid cells. It is important in the regulation of the inflammatory state of myeloid cells. Suppressive myeloid cells are involved in inhibition of anti-tumor immunity as well as in prevention of immune cell infiltration into the tumor microenvironment. These mechanisms of myeloid cell action reduce the response to immune checkpoint blockade therapy. However, deletion of IRAK3 in immunocompetent mice has led to not only the inhibition of tumor growth but also a synergy with immune checkpoint blockade to improve its therapeutic effect (Tunalı et al., 2023).

Aims

Since the importance of NEDD8 has been previously shown in the resistance to ICB it is worthwhile to investigate how other components of the neddylation pathway are involved in cancer cell sensitivity to therapy. Furthermore, testing of how the inhibition of these pathway components affects cell viability and proliferation.

As previously mentioned, pevonedistat is an inhibitor of the NEDD8-activating enzyme, however data has shown that it might also have other intracellular targets that are not well understood. Here, the aim is to understand the importance of NAE1 both to the cell and to killing induced via pevonedistat.

Finally, we sought out to investigate whether genetic deletion of IRAK3 in a host organism had a synergistic effect with genetic deletion of NEDD8 in a cancer cell line in terms of tumor growth and development.

Materials and methods

Cell lines used and cell culture

The two cell lines used were the human triple negative breast cancer cell line MDA-MB-231 obtained from American Type Culture Collection (ATCC) as well as the murine breast cancer cell line EO771, originally obtained from Dr. Maria Ulvmar (Uppsala University, Sweden).

Both cell lines were maintained in Iscove′s Modified Dulbecco′s Medium (IMDM) cell culture medium (gibco, UK, CAS No. 21980-032) with added 10% heat-inactivated fetal bovine serum (gibco, Germany, CAS No. 10500-064) and 1% penicillin-streptomycin (gibco, CAS No. 15140-122). Cells were kept in incubators at 37°C with 5% CO2. Cells were passaged every 3-4 days and checked for mycoplasma infection routinely.

Tumor Immune Co-Culture System

Day 1 of the experimental procedure centred around harvesting of cancer cells using the standard procedure. This consisted of first removing culture media from cell-containing flasks. Next, cells were briefly washed with 10 ml phosphobuffered saline (PBS) (gibco, UK, CAS No. 10010-023) which was then removed and 3 ml Versene (gibco, USA, CAS No. 15040-066) was added. Cells were incubated at 37°C for 5 min. Each flask was tapped on the walls to dislodge cells and then washed a few times with PBS. The suspension was then transferred into 15 ml falcon tubes and centrifuged at 0.6 g for 3.5 min. Then, cells were resuspended to 0.1 million cells/ml. Following this, cells were transferred to a 96-well flat bottom plate (Corning, CAS No. 353072) at a final number of 10 000, 5000, 2500, 1250 in duplicates for each treatment condition. One more set of well duplicates was used as a control with no added cancer cells. The plate was then briefly pulse centrifuged to allow the cells to settle in the plate. The plate was incubated at 37°C overnight.

On day 2, in order to isolate primary human lymphocytes, frozen peripheral blood mononuclear cells (PBMCs) that were isolated from healthy donors (Uppsala University Hospital, Sweden) were used. The PBMCs were thawed at 37°C and transferred into a 15 ml falcon tube. Then, cells were washed in the tube using 13 ml PBS and centrifuged at 0.6 g for 3.5 min. Washing and centrifuging was repeated once. For every one million of cells, 1 µl of anti-CD14 antibodies was added to the tube (StemCell, Easysep Human CD14 positive cocktail, CAS No. CAS No.

17858C). The sample was incubated for 10 min at room temperature and then vortexed briefly. Next, for every one million of cells, 1 µl of Rapid spheres was added to the tube (StemCell, CAS No. 50100). The sample was then incubated for 3 min at room temperature.

Next, two volumes of PBS were added to the sample tube, which was then placed into an EasySep magnet (StemCell, CAS No. 18103) and incubated for 8 min at room temperature. After incubation, the supernatant that remains was carefully collected and transferred into fresh falcon tubes.

Isolated lymphocytes were then counted and resuspended to a concentration of 3 million cells per ml of medium. The cells were equally divided in falcon tubes, one tube was kept as a control while immune checkpoint blockade antibodies (anti-PD-1 and anti-PD-L1) were added to the other tubes. Next, 100 µl of lymphocyte samples was added to the corresponding wells containing cancer cells. The co-culture plate was incubated at 37°C for 5 days. Supernatants were harvested and transferred into a 96-well V-bottom plate (Corning, CAS No. 3897). The plate was centrifuged at 700 g for 3 min. Finally, 150 µl of supernatant was transferred into a 96-well U-bottom plate (Corning, CAS No. 3879) and stored at -20°C for further use in Enzyme-Linked Immunosorbent Assays.

Enzyme-Linked Immunosorbent Assay

ELISA was used for analysis of immune activation molecules in supernatant collected from the TICS assay. The manufacturer's protocol was used for this purpose: granzyme B (Mabtech, ELISA flex: Human Granzyme B (HRP), CAS No. 3486-1H-6.

Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR) Cas9 gene deletion

The CRISPR Cas9 system was used for the generation of gene knockout cell lines. For this purpose, CRISPR ribonucleoprotein (RNP) complexes were prepared. For the knockout, the following was combined in a PCR tube: 1.7 µl Nuclease-Free Duplex Buffer (IDT, CAS No. 11-01-03-01), 1 µl (100 µmol) tracrRNA, 1 µl (100 µmol) crRNA (Table 1). In the control tube, no crRNA was added, instead an equal volume of IDTE buffer pH 7.5 (IDT, CAS No. 11-01-02-02) was added. PCR tubes were then placed in a thermocycler and run at 95°C for 5 min followed by 4°C incubation.

The following part of the process began with the dilution of endonuclease Cas9 10x in buffer R (ThermoFisher, CAS No. BR5). Diluted Cas9 (1 µl, 10 mg/ml) was added to each reaction tube that contained RNP complex, and incubated for 15 min. Then, carrier DNA (0.3 µl, 100 µM) (Table 1) was added to the reaction tubes. Simultaneously, the Neon transfection system (Invitrogen, Catalog No. MPK5000) was set up. Next, cells were harvested using the standard procedure previously mentioned. Cell pellet ($5x10^5$ cells) was carefully resuspended with an equal volume of buffer R and RNP complex using a transfection system pipette. Finally, the suspension was placed into the transfection system pipette holder and an electroporation program was run. Following this step each sample was transferred into a corresponding well in a 12 well plate (Corning, CAS No. 353043) with growth media. All cells were kept in a regular cell culture incubator overnight and transferred into cell culture flasks the following day. To achieve complete gene deletion, gene-targeting, or control RNP complexes were repeatedly transfected to cells.

Table 1: Nucleotide sequences used for CRISPR Cas9 gene deletion

	Nucleotide sequence
Carrier DNA	5'-CCA GCA GAA CAC CCC CAT CGG CGA CG-3'
crRNA 2	5'-/AltR1/rUrArUrGrUrCrCrUrArCrArGrArUrC-3'
crRNA 3	5'-/AltR1/rUrCrArArArGrArArGrCrArGrUrArU-3'

Western blot

Cell pellets were lysed using RIPA buffer (ThermoFisher Scientific, CAS No. 89900) and protease inhibitor (ThermoFisher Scientific, CAS No. 1861278). Following lysis buffer addition, samples were incubated in ice for 15 min. Samples were then centrifuged at 17 g for 10 min at 4°C and the supernatant was collected and transferred into fresh Eppendorf tubes.

To measure protein concentration in the samples the Pierce™ BCA Protein Assay Kit (ThermoFisher, CAS No. 23225) was used based on the manufacturer's instructions. BSA standard at differing concentrations were used to determine the concentration of protein in the samples. Once the protein concentrations were measured a calculated volume of each sample

was mixed with lysis buffer and loading dye (NuPAGE LDS Sample Buffer (4X), ThermoFisher, CAS No. NP0008), followed by incubation at 70°C for 12 min.

After preparation of running buffer (NuPAGE MOPS SDS Running Buffer (20X) Invitrogen, CAS No. NP0001) samples were loaded on the 4-12% SDS-PAGE gel (ThermoFisher, CAS No. NP0336BOX). Samples were loaded onto the gel along with the PageRuler™ Plus Prestained Protein Ladder (ThermoFisher Scientific, CAS No. 26619). Electrophoresis was run at 140 V for approximately 1 hour. Then, proteins were transferred from the gel to a nitrocellulose membrane using iBlot™ 2 Transfer Stacks, (Invitrogen, CAS No. IB23002 or ThermoFisher Scientific, Catalog no. IB21001) and the iBlot™ 2 Gel Transfer Device. After transfer was completed, the membrane was briefly incubated with Ponceau S Solution for Electrophoresis (0.2 %) (SERVA, CAS No. 33427.01) to visualize protein bands. Next, the membranes were blocked with 5% nonfat SKIM milk powder (OXOID) followed by incubation, which was followed by the addition of primary antibodies- NAE1 (Proteintech, CAS No. 14863-I-AP), NEDD8 (Cell Signal Technologies, CAS No. 2754S) and incubation at 4°C overnight.

On day 2, the membrane was incubated with HRP-linked secondary antibody- anti-rabbit IgG (Cell Signaling Technology, CAS No. 7074S) or anti-mouse IgG (Cell Signaling Technology, CAS No. 7076S), and incubated for 1 hour at room temperature. To visualize the bands during imaging, SuperSignal™ West Pico PLUS Chemiluminescent Substrate (ThermoFisher Scientific, CAS No. 34580) was used and the GE Amersham 680 Imager. After each step the membranes were washed with TBST (1X TBS, 0.05% Tween 20, dH2O). Vinculin (Sigma, CAS No. V9131) was used as a loading control.

IncuCyte Live Cell Analysis

On the first day of experiment, cells were harvested using the standard procedure previously mentioned. Once harvested, the cells were counted for a wanted concentration of 5000 cells per well of a 96 well flat bottom plate at a total volume of 96 µl (Corning, CAS No. 353072). After plating, cells were incubated in a cell culture incubator overnight in order to adhere to the microplate surface.

On the second day, pevonedistat (Selleckchem, CAS No. 905579-51-3) was diluted in series with dimethyl sulfoxide (DMSO) (Fisher Scientific, CAS No. 67-68-5) to final concentrations of 5000

nM, 3000 nM, 1000 nM, 800 nM, 500 nM, 300 nM, 100 nM, 30 nM, 10 nM, and 1 nM. Once diluted, an equal volume of inhibitor was added to corresponding cell-containing wells, while the last well of each condition had only DMSO added that acted as a growth rate control.

The plate was then incubated in the incucyte image zoom system (Essenbioscience, Catalog No. 9500-4459) and imaging was conducted at indicated time points to evaluate cancer cell proliferation. The data collected was then processed in GraphPad Prism software (Dotmatics, version 9.0.0).

***In vivo* work**

The study was carried out using 31 immuno-competent female mice. Two strains of mice were used, C57BL/6NTac (obtained from Taconic Biosciences) (16 mice), and IRAK3KO (15 mice) were obtained from the animal facility at the department of Immunology, Genetics, and Pathology, Uppsala University. The mice were injected subcutaneously with $6x10^5$ EO771 NEDD8 KO cells. They were further separated into groups that received a PBS injection or an injection containing 50 µg of anti-PD-1 antibody (BioCell, InVivoMAb, CAS No. BE0146). The mice were injected intra-peritoneally with treatment and control on days 7, 10, and 13 post tumor injection. All mice were checked three times a week, and once tumors were established, they were measured upon each check, whereas bodyweight was measured once per week. Once the tumors had reached a sufficient size, tumors and spleens were collected for fluorescence-activated cell sorting analysis.

Pevonedistat (MLN4924) resistant cell line generation

For the purpose of generating a drug resistant cell line the EO771, parental cell line was used. Cells were harvested and counted using the standard procedure. Then $5x10^5$ cells were added per well in a 6 well flat bottom plate (Corning, CAS No. 353046). Next, the cells were treated with pevonedistat at a concentration of 3000 nM, or 5000 nM. Once cells reached 65-70% confluency, they were harvested and seeded onto a fresh 6 well plate with the same cell number and treated with inhibitor in the same manner. This development period lasted for approximately 1 month. The sensitivity of resistant cells to pevonedistat was determined using the incucyte zoom live imaging system, as described above. This resistant subline was stored at -80°C until use.

Results

Pevonedistat resistance as a proof of concept for targeting neddylation

Since pevonedistat resistance leads to stronger activation of the neddylation pathway the natural question was to investigate how this affected immune cell activation compared to wildtype cells. For this purpose, a TICS assay was performed which showed that pevonedistat resistant cells led to a much lower secretion of soluble granzyme B in untreated cells (Figure 4).

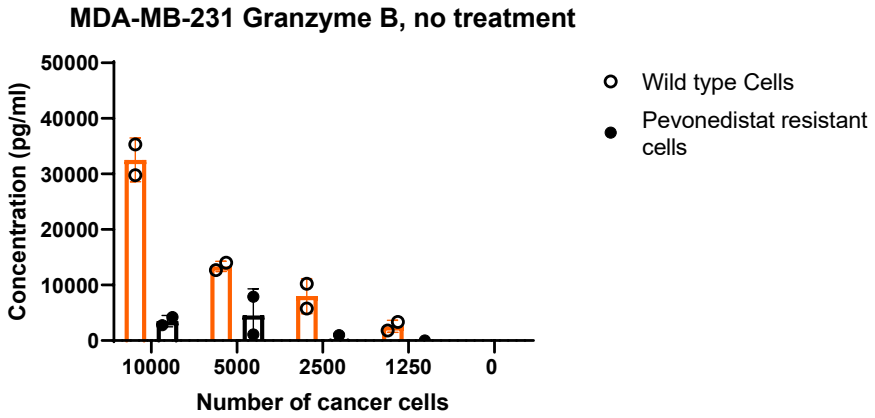

Figure 4: Pevonedistat resistant cells lead to lower granzyme B secretion in co-culture. Wildtype or pevonedistat resistant MDA-MB-231 cells were co-cultured with primary human lymphocytes for 5 days after which ELISA was performed to test for secretion of soluble granzyme B

Moreover, co-cultured cells that were treated with anti-PD-1, or anti-PD-L1 ICB therapy also maintained the trend of lower soluble granzyme B secretion in resistant cells with a markedly small increase in granzyme B secretion with resistant cells at 10^3 cancer cells with anti-PD-L1 therapy (Figure 5).

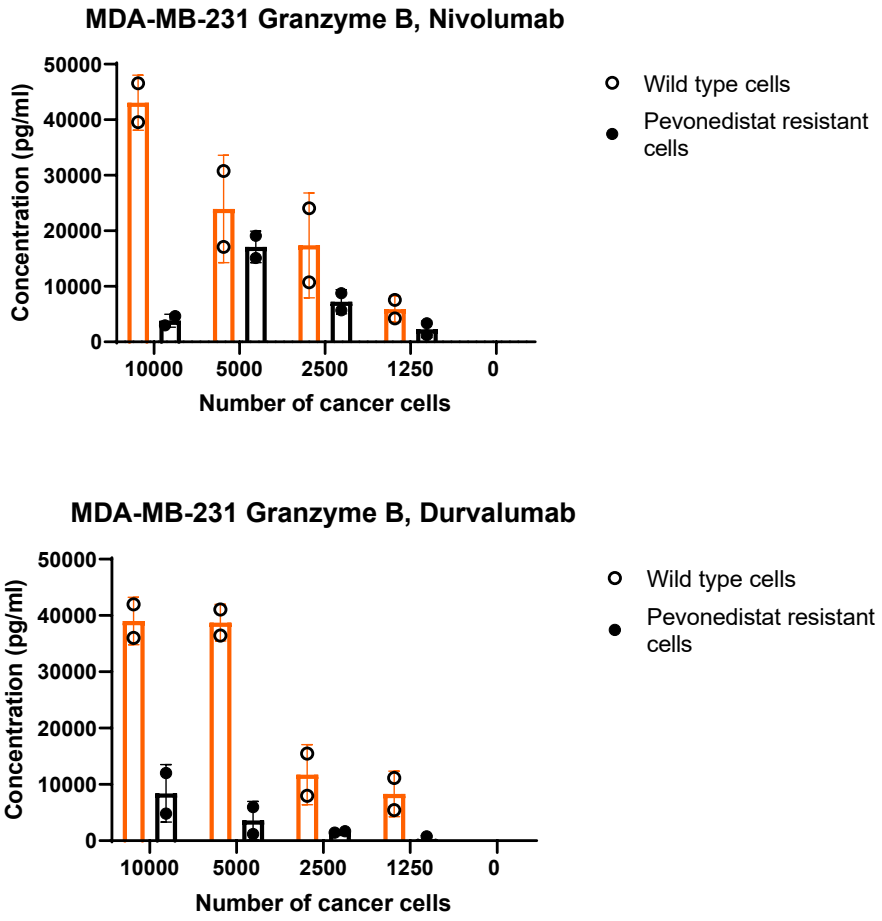

Figure 5: Pevonedistat resistant cells treated with ICB therapy in an immune co-culture show much lower granzyme B secretion compared to wildtype cells. Wildtype or pevonedistat resistant MDA-MB-231 cells were co-cultured with primary human lymphocytes with either 10 µg/ml of anti-PD-1 (nivolumab) or 10 µg/ml anti-PD-L1 (durvalumab) for 5 days after which ELISA was performed to test for secretion of soluble granzyme B

NAE1 knockdown does not affect pevonedistat cell killing

Gene knockdown (KD) cells were generated using the CRISPR Cas9 platform to gain a better understanding of the neddylation pathway, how different components affect cell viability and proliferation, as well as how this knockdown changes inhibitor tolerance. The target chosen was NAE1, the regulatory subunit of the NEDD8 E1 enzyme, the very same enzyme where pevonedistat binds. Once a knockdown was achieved (Figure 6), cells were cultured, and an IncuCyte live cell analysis assay was carried out.

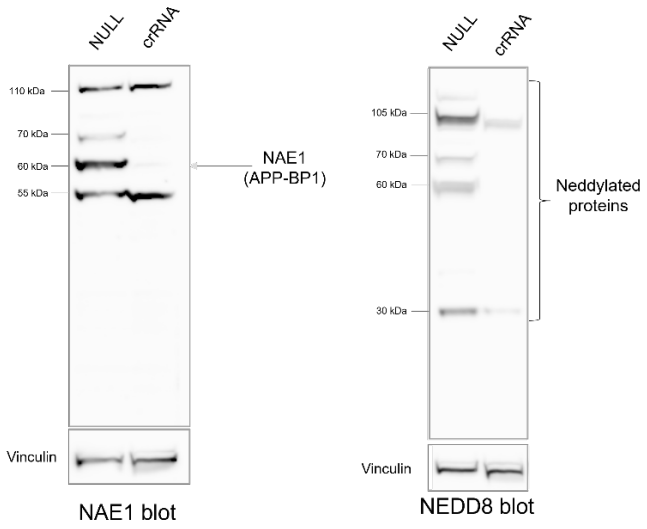

Figure 6: Knockdown of NAE1 leads to changes in neddylation. Expression of NAE1 was measured using Western blot after several rounds of transfection using the CRISPR Cas9 platform. The right side of the figure shows the expression of NEDD8 and neddylated proteins. Vinculin was used as a loading control

Surprisingly, the IncuCyte live cell analysis showed that even though NAE1 was knocked down, pevonedistat continued to kill cells in very much the same manner as with wildtype cells (Figure 7).

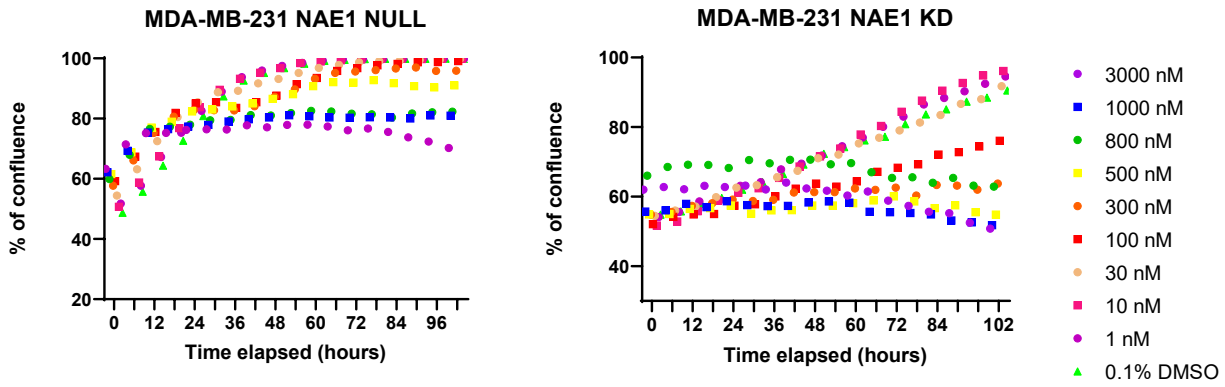

Figure 7: Pevonedistat induces cell death in NAE1 knockdown cells in a similar manner compared to null cells. Proliferation of null and knockdown cells treated with varying concentrations of inhibitor was monitored using a live cell imaging system

Generation of a murine pevonedistat resistant cell line

Pevonedistat resistant murine breast cancer cells (EO771) were generated by continual treatment of cultured cells over the period of about 1 month as described in the materials and methods section. In order to confirm that pevonedistat treated cells were resistant to the neddylation inhibitor an IncuCyte live cell analysis was carried out. Resistant cells showed a consistent resilience to pevonedistat compared to control cells (Figure 8).

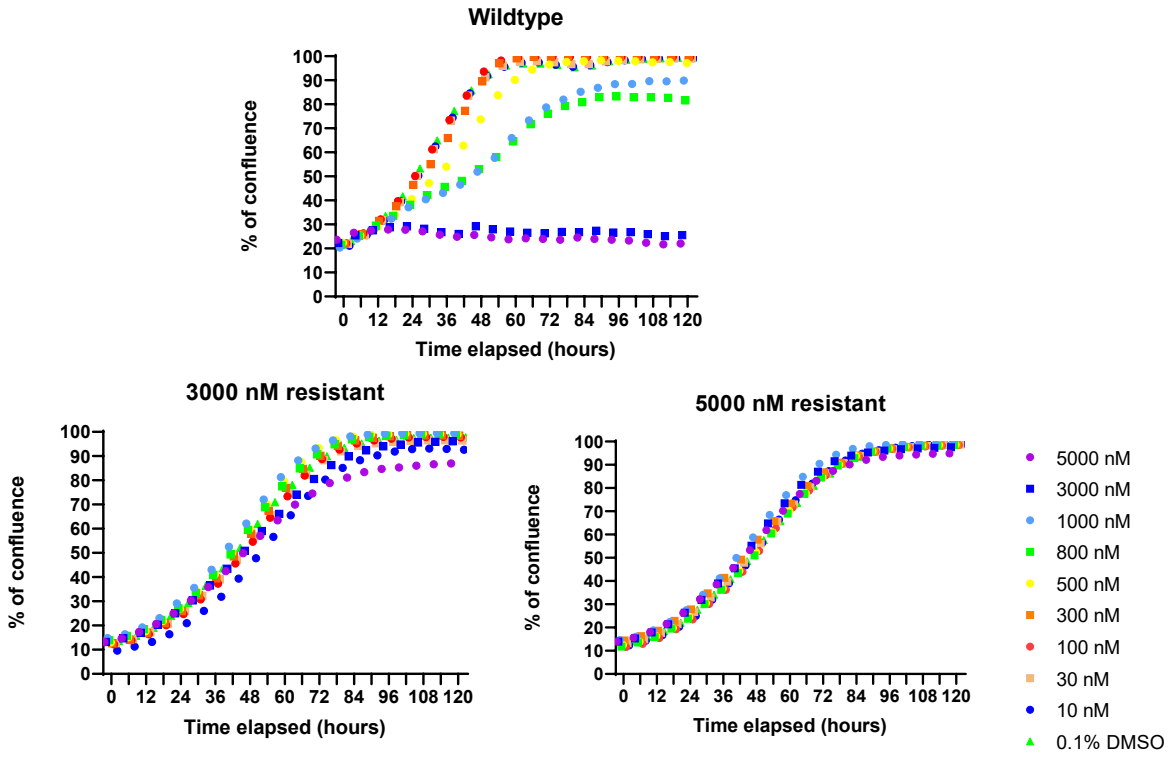

Figure 8: Conditioned cells show a sustained resistance even at high concentrations of inhibitor. Wildtype and resistant cells were treated with differing concentrations of inhibitor and analyzed using a live cell imaging system to investigate whether or not resistant cell lines were successfully generated

The only notable difference between resistant and control cells was that resistant cells grew at a slightly slower rate (Figure 9), something that did not affect the overall difficulty in culturing and maintaining these cells.

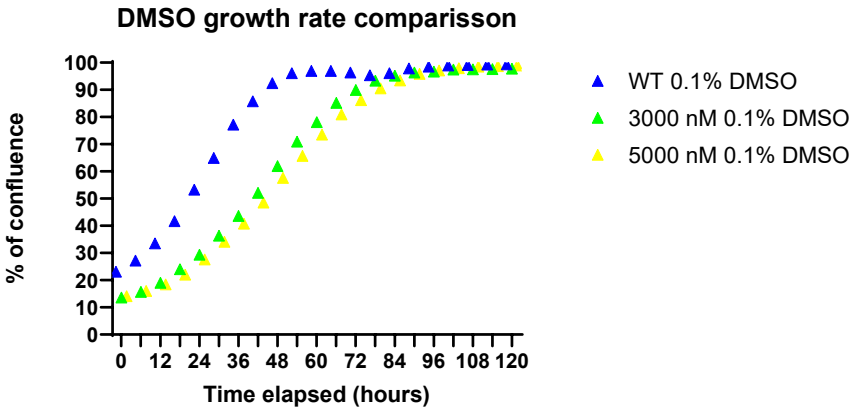

Figure 9: Resistant cells grow at a slightly slower rate compared to parental wildtype cells. Growth rate was compared between wildtype and resistant cells using the DMSO control during live cell imaging

IRAK3 knockout mice have lower average tumor size compared to wildtype

All mice in this study were injected with NEDD8 KO cancer cells (EO771). The mice were divided into four groups: wildtype mice treated with PBS, wildtype mice treated with anti-PD-1 ICB therapy, IRAK3 KO mice treated with PBS, and IRAK3 KO mice treated with anti-PD-1 ICB therapy. Tumor growth in IRAK3 KO mice with or without anti-PD-1 treatment was, on average, comparable to tumor growth in wildtype mice treated with anti-PD-1 (Figure 10). Wildtype mice treated with PBS had the largest tumor volumes (Figure 10).

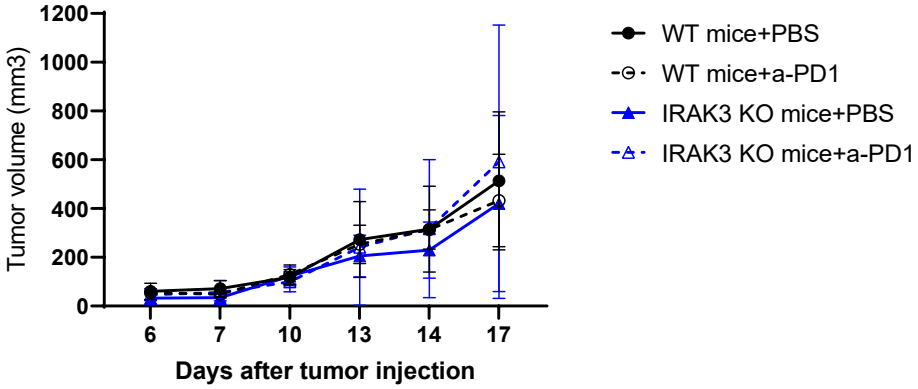

Figure 10: Tumor volume comparison between the four experimental groups showed slight differences. Six hundred thousand EO771 NEDD8 KO cells were injected subcutaneously into WT mice of the C57BL/6NTac background and IRAK3 KO mice (6-10 weeks old). Mice were injected intraperitoneally with either PBS or 50 µg of anti-PD-1 ICB on days 7, 10, and 13 post tumor injection. Once palpable, tumor sizes were checked and measured 3 times per week and tumor sizes were compared once the first mice were removed due to a large tumor volume

Discussion

Neddylation has been presented as an important factor not only in cancer cells but in the tumor microenvironment (TME) as well (L. Zhou et al., 2019). Cancer-associated fibroblasts, a key component of the TME, are significantly impacted by the neddylation pathway. Inhibition of neddylation using pevonedistat led to downregulation of genes required for cell cycle progression and DNA replication (L. Zhou et al., 2019). Conversely, neddylation is also a key player in the regulation of T-cells. It is required for $CD4^+$ T-cell proliferation, activation, and survival (Zou & Zhang, 2021). Treatment of T-cells with pevonedistat led to impaired cytokine secretion, as well as cell cycle arrest (Biedermann et al., 2004; Jin et al., 2013). Use of pevonedistat or methods such as siRNA for silencing of other components of the pathway caused reduction in proliferation of thymic epithelial cells and consequent reduction in thymus size. It also had overarching diminishing effects on Th1 and Th2 cells, downregulation of Tregs as well as a reduction of TNFα and IL6 cytokine production across different immune cell types (Y. Lu & Yang, 2020).

In cancer, however, the neddylation pathway facilitates cell survival across multiple types by processing mutated, misfolded proteins (McGrail et al., 2020). In contrast, suppression of different components of the neddylation pathway resulted in an upregulation of PD-L1 mRNA levels along with increased cell surface levels of PD-L1. Due to this observation, a combination of pevonedistat and anti-PD-1/anti-PD-L1 ICB therapy was investigated. The combination showed a strong synergistic effect towards immune-mediated cell killing. ICB in this combination served as a solution in overcoming pevonedistat-induced immune resistance caused by the upregulation of PD-L1 (S. Zhou et al., 2019). Another study, by McGrail et al., demonstrated that pevonedistat treatment of microsatellite instable tumors caused immunogenic cell death by potentiating expression of cytokines linked with $CD4^+$ T-cell recruitment. This line of research further demonstrated a strong synergy between pevonedistat and anti-PD-1 therapy, although, this effect was limited to tumors with a DNA mismatch repair deficiency (McGrail et al., 2020).

Since this large body of data supports the notion of a synergistic effect between pevonedistat and ICB therapy, my research group sought to investigate what made cancer cells resistant to anti-

PD-1 ICB in the first place. Results showed that NEDD8 itself was the most probable resistance gene for this type of therapy (I. Papakyriacou, unpublished data). Because of this, research here focused on further looking into the effects of pevonedistat, NEDD8, and other components of the neddylation pathway.

Pevonedistat resistance in cancer cells clearly leads to an overactivation of the neddylation pathway (I. Papakyriacou, unpublished data) as well as to much lower secretion of soluble granzyme B (Figure 4), an indicator of immune activation. However, IncuCyte live cell analysis experiments on both NAE1 KD (Figure 7) and NEDD8 KO (I. Papakyriacou, unpublished data) cells treated with pevonedistat led to an equal amount of cell killing compared to wildtype cells. This raises questions as to the actual mechanism of pevonedistat-induced killing. Pevonedistat is said to be a selective NAE inhibitor (Torka et al., 2020), yet cells with gene deletions of both NAE1, one of the two proteins that make up the cleft for pevonedistat binding, and NEDD8, the centerpiece of the neddylation pathway survive and continue to proliferate. Yet, upon treatment with pevonedistat these cells die (Figure 7). Clearly, this shows that pevonedistat has other targets in the cell which are yet to be understood.

Generating a gene deletion of NAE1 tried to accomplish two goals: further understanding of pevonedistat-induced cellular killing, as well as of cellular behavior with manipulation of differing components of the neddylation pathway. Indeed, NAE1 has proven to be quite the challenge in regard to complete gene deletion. Once a knockdown of the gene is achieved cells grow at a significantly lower rate compared to null cells (Figure 6-7), which makes it impossible to conduct other experiments at this time. When cells eventually reach a number which is high enough for use in other assays, they enter a period of approximately 3 days of sustained knockdown after which gene expression comes back. This has been demonstrated continually over several rounds of CRISPR Cas9 gene deletion, where the same end result was observed every time (Figure S1). There could be several reasons for this. NAE1 has been proven to be an essential gene, specifically in the MDA-MB-231 cell line (depmap.org). The importance of NAE1 can be further argued due to its interaction with amyloid precursor protein (APP) which has implications for cell cycle progression. It has been previously demonstrated that depletion of NAE1 in fetal neural stem cells leads to cell cycle arrest in G1 phase. Moreover, APP binding

acts as an antagonist upon binding with NAE1, although it is believed that this serves as a control mechanism to prevent over proliferation of cells (Joo et al., 2010).

However, not a lot of research has been conducted concerning the specific roles of NAE1 and further work is required to understand the intricacies of NAE1 function in the cell.

Previous research demonstrated that treatment of tumors with anti-PD-1 therapy in IRAK3 KO mice resulted in consistently small tumor size and prolonged animal survival. Apart from this, a significant increase in the number of $CD4^+$ and $CD8^+$ T-cells was observed, as well as an increase in activation of dendritic cells (Tunalı et al., 2023).

Since NEDD8 KO cell lines have been demonstrated as sensitive to anti-PD-1 blockade previously (I. Papakyriacou, unpublished data), we set out to investigate whether there was a synergistic effect between an IRAK3 KO host and NEDD8 KO tumor cells +/- anti-PD-1 ICB therapy. The experimental setup entailed the use of knockout cells in all groups, while there was a divide made between wildtype mice and IRAK3 KO mice. Mice were further split between mock and anti-PD-1 therapy receiving groups as described in materials and methods.

As expected, wildtype mice with mock treatment developed the largest tumors on average, compared to the other groups (Figure 10). On the other hand, there was no major difference between the remaining groups. From day 17 post tumor injection a regression in tumor size was observed in both IRAK3 KO groups while tumors in wildtype mice continued to grow at the same rate regardless of treatment condition. Finally, it was not possible to measure tumors in IRAK3 KO mice due to their small size and the depth at which they grew. The rest of the IRAK3 KO mice in both treatment groups had tumors that kept growing, albeit still at a smaller size compared to wildtype mice. Questions remain about what influenced tumor growth as well as subsequent regression in size, but this will be further evaluated in the future.

With the end goal of gaining a better understanding of the effects of pevonedistat resistance *in vivo,* the murine breast cancer cell line EO771 was made resistant (Figure 8). These cells aim to show the implications of an overactive neddylation pathway with ICB therapy as well as the effect this will have on immune activation in the host. It can be hypothesized that these tumors would be resistant to treatment to a high extent, but it remains to be seen what will happen in a live animal model.

Building on research already done here, several points remain to be addressed. First off, a study is planned to further understand the mode of cellular killing induced by pevonedistat treatment. This will utilize a CRISPR screen for the identification of depleted and enriched genes in cells upon treatment. For this purpose, a stable knockdown or knockout must first be established in MDA-MB-231 cells. Different approaches with the CRISPR Cas9 platform will be explored. Chemoproteomics analysis will also be performed to get a clearer understanding of where pevonedistat binds within the cell.

Next, an *in vivo* study will be carried out using the generated pevonedistat resistant murine cancer cell line.

Finally, it remains to be seen what kind of a relationship is established between NEDD8 KO cancer cells and IRAK3 KO mice. FACS analysis of tumor and spleen samples will, hopefully, shed light on this and provide more information as opposed to tumor volumes alone.

A deeper understanding of the neddylation pathway has potential to provide insight into therapy resistance in triple negative breast cancers and how tumors can potentially be resensitized to treatment.

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Finally, I would like to dedicate this thesis to my dad, who sadly passed away during the first year of my master's studies. He always pushed me to strive for bigger things, and I hope that this would make him proud.

Once again, thank you all!

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Supplemental data

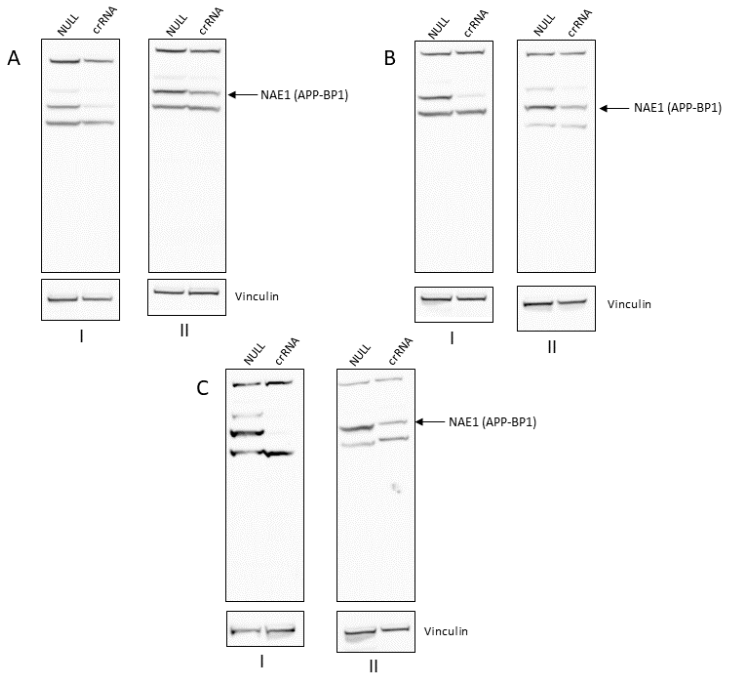

Figure S1: NAE1 expression returns to normal levels several days post transfection, across multiple rounds of CRISPR *Cas9* gene deletion. **A**: Western blots for the third round of gene deletion carried out with cell pellet collected on days 4 (I) and 23 (II) post transfection. **B**: Western blots for the fifth round of gene deletion carried out with cell pellet collected on days 7 (I) and 14 (II) post transfection. **C**: Western blots for the sixth round of gene deletion carried out with cell pellet collected on days 7 (I) and 15 (II) post transfection