European Journal of Immunology
Innate immunity
Basic

Research Article

The Ubiquitin-proteasome pathway regulates Nectin2/CD112 expression and impairs NK cell recognition and killing

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Nectin2 is a member of immunoglobulin-like cell adhesion molecules and plays a prominent role in the establishment of adherens and tight junctions. It is also upregulated on the surface of tumor and virus-infected cells where it functions as a ligand for the activating receptor CD226, thus contributing to cytotoxic lymphocyte-mediated recognition and killing of damaged cells. Little is currently known about the regulation of Nectin2 expression and, in particular, whether posttranscriptional and posttranslational mechanisms are involved. Here, we analyzed Nectin2 expression on a panel of human tumor cell lines and primary cultures and we found that Nectin2 is mainly expressed in cytoplasmic pools. Moreover, we demonstrated that ubiquitination of Nectin2 promotes its degradation and is responsible for protein intracellular retention. Indeed, inhibition of the ubiquitin pathway results in increased Nectin2 surface expression and enhances tumor cell susceptibility to NK cell cytotoxicity. Our results demonstrate a previously unknown mechanism of Nectin2 regulation revealing that the ubiquitin pathway represents a potential target of intervention in order to increase susceptibility to NK cell-mediated lysis.

Keywords: Immune surveillance · Innate immune system · NK cell receptors · Posttranslational modification · Ubiquitination

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Introduction

Nectin and Nectin-like molecules are adhesion proteins belonging to the Ig superfamily. Both the four members of the Nectin family (Nect1-4) and the five members of the Nectin-like family (Necl1-5) play a fundamental role in the formation of adherens and tight junctions between epithelial cells and fibroblasts as well as synaptic junctions of neurons, by forming homo or heterodimers with proteins typically belonging to the same family [1, 2]. Some members of Nectin and Nectin-like proteins are also capable of binding molecules other than Nectins, thus mediating a wide range of physiological processes including cell polarization, cell movement, proliferation, and immune modulation as well as pathological processes such as cancer progression and host-pathogen interactions [3, 4]. In particular, Nectin-like5 (CD155), also known as poliovirus receptor (PVR), was originally identified as entry receptor for the Poliovirus [5], while Nectin1 (CD111),

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and Nectin2 (CD112) mediate the entry of herpesviruses into their target cells [6, 7].

Nectin2 and PVR also regulate immune responses thanks to their ability of interacting with structurally related immune receptors, namely CD226 (DNAM1, DNAX-associated molecule1), CD96, TIGIT [3, 4], and the recently identified inhibitory receptor CD112R [8].

The activating receptor CD226 is expressed on several immune cells including NK cells, monocytes, and different T lymphocyte subsets (CD4+ and CD8+, NKT and $\gamma\delta$ T cells). CD226 binds to both Nectin2 and PVR [3, 9, 10] and plays a role as a molecule mediating adhesion/migration processes [11]. On NK cells and cytotoxic CD8+T lymphocytes (CTL), CD226 contributes to the recognition and killing of transformed and virus-infected cells [12–15]. Accordingly, CD226 knockout mice support a pivotal role for this receptor in anti-tumor and anti-viral immune surveillance [13, 14].

Both Nectin2 and PVR expression is upregulated on tumor and virus-infected cells [16–28], leading to an increased CD226-mediated recognition.

Thus, the molecular mechanisms underlying upregulation of CD226 ligands is a field of interest based on the possibility to potentiate anti-tumor and anti-viral immune responses.

Little is known about the involvement of posttranslational modifications that could provide a rapid way to regulate CD226 ligand expression. We have recently demonstrated that both PVR and Nectin2 are mainly expressed as intracellular pool in the haematological malignancy multiple myeloma (MM), and that a posttranslational modification of PVR, namely SUMOylation, regulates its subcellular localization [29]. However, the mechanism involved in Nectin2 intracellular retention is still unknown.

Ubiquitination, i.e. the covalent addition of one or several Ubiquitins (Ubs) on a target protein, is a posttranslational modification involved in several aspects of eukaryotic biology [30, 31]. Ubiquitinated substrates may undergo proteasome-dependent degradation as well as nondegradative fate, including, for membrane proteins, endocytosis, and intracellular vesicle trafficking [30, 32].

Here, we show that Nectin2 is subjected to ubiquitination, which is responsible for Nectin2 proteasomal degradation and protein retention in intracellular compartments. The inhibition of the Ub pathway promotes an upregulation of Nectin2 surface expression on tumor cells that results in increased target cell susceptibility to NK-mediated cytolysis.

Collectively, our data elucidate a new molecular mechanism that regulate the expression of Nectin2, revealing that the Ub pathway can be targeted to increase NK-cell capability to kill tumors and virus-infected cells.

Results

Nectin2 is mainly expressed as intracellular pool

We firstly analyzed Nectin2 expression by immunofluorescence and FACS analysis on a panel of tumor cell lines before and after permeabilization to evaluate surface and total (surface plus intracellular) protein levels, respectively, indicating that Nectin2 is mainly present in intracellular compartments (Fig. 1A). Of note, Nectin2 intracellular retention was also observed on primary cultured human foreskin fibroblasts (HFF) and HUVEC (Fig. 1B).

These results demonstrate that the presence of an intracellular Nectin2 pool is a common feature shared by several types of cancer as well as normal cells and suggest that a posttranslational mechanism of membrane/cytosol distribution controls Nectin2 surface expression.

The Ub pathway affects Nectin2 expression and impairs target cell susceptibility to NK cell killing

Ubiquitination and SUMOylation represent the posttranslational mechanisms most likely implicated in the control of protein expression due to their well-known role in the regulation of protein conformation/stability and subcellular localization [30, 31, 33, 34].

Since we have previously excluded the involvement of SUMO pathway in the regulation of Nectin2 expression [29], we focused our attention on the Ub pathway. We treated ARK MM and HeLa cervical carcinoma cell lines with PYR41, a specific inhibitor of the E1 activating enzyme responsible for protein ubiquitination [35], and the surface expression of CD226 ligands was evaluated by flow cytometry. PYR41 treatment induced Nectin2 but not PVR upregulation on ARK cells (Fig. 2A), suggesting that the Ub pathway selectively regulates Nectin2 expression.

To investigate whether Nectin2 surface upregulation renders target cells more efficiently recognized and killed by NK cells, we performed a cytotoxicity assay in which primary cultured human NK cells were used as effector cells. Increased NK cell cytotoxicity was observed against PYR41-treated ARK cells in respect to untreated cells at all effector:target cell ratios analyzed (Fig. 2B). This increase was abrogated in the presence of an anti-CD226 antibody that interferes with Nectin2-CD226 interaction, strongly suggesting that Nectin2 upregulation is responsible for the increased ARK cell susceptibility to NK cell lysis.

Similar, although less pronounced, results were obtained using HeLa cells: treatment with PYR41 induced an upregulation of Nectin2 surface expression and increased susceptibility to NK cell lysis (Fig. 2C and D).

All together our results demonstrate that the inhibition of the Ub pathway increases Nectin2 surface expression on tumor cells and render them more susceptible to CD226-dependent NK cell cytotoxicity.

Nectin2 is subjected to ubiquitination

To investigate whether Nectin2 undergoes ubiquitination, the protein was immunoprecipitated from ARK and HeLa cells. Western blot analysis with anti-Ub antibody reveals the presence of slower migrating molecular species that specifically coprecipitated with

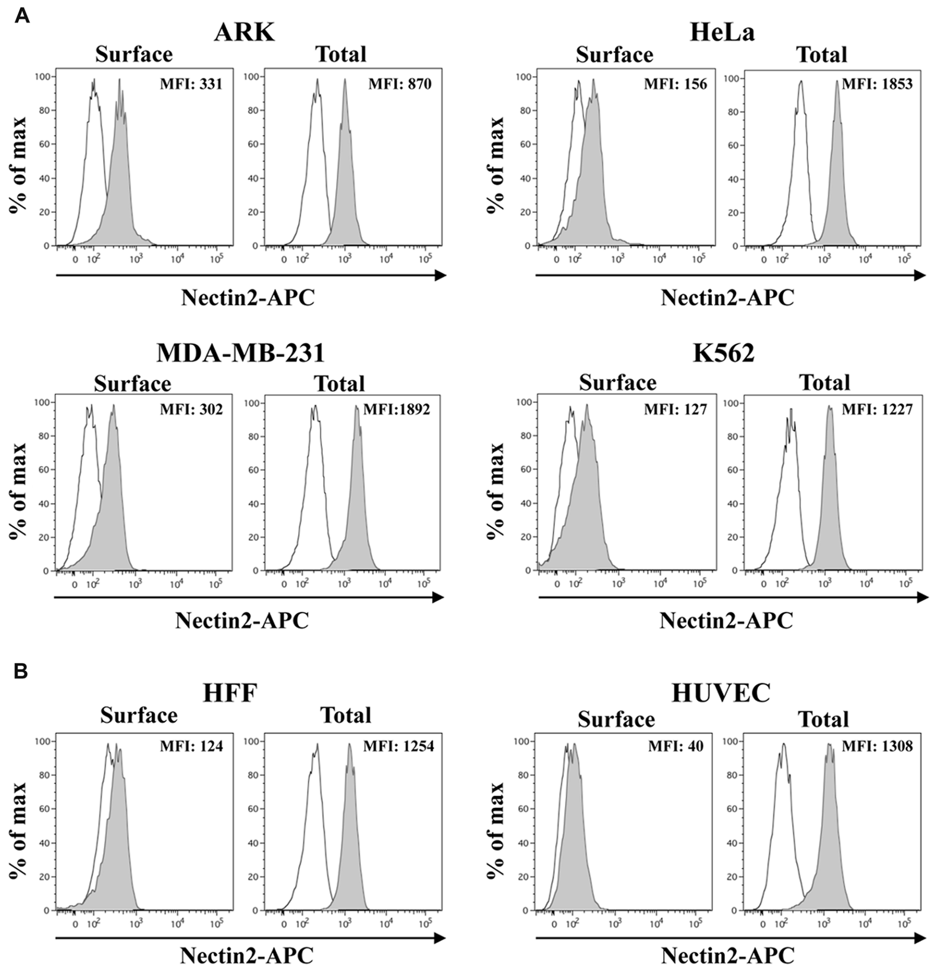

Figure 1. Nectin2 is mainly expressed as intracellular pool. (A and B) Nectin2 surface and total protein levels were analyzed on the indicated cell lines before and after fixation and permeabilization, respectively. Cells were acquired using FACSCanto flow cytometer using the strategy illustrated in Supporting Information Fig. 3. Empty histograms represent APC-conjugated isotype-matched control Ig; full histograms represent APC-conjugated anti-Nectin2 staining. The MFI of Nectin2, obtained by subtracting the MFI of the isotype control Ig, was indicated in each panel. One representative out of three independent experiments is shown.

Nectin2 in both cell lines (Fig. 3A), suggesting that they correspond to ubiquitinated Nectin2 species.

To demonstrate that Nectin2 is directly ubiquitinated, we performed proximity ligation assay (PLA). ARK and HeLa cells were incubated with polyclonal anti-Ub alone (as negative control) or in combination with monoclonal anti-Nectin2, subjected to PLA amplification protocol, and analyzed by confocal microscopy (Fig. 3B). We observed the appearance of PLA positive spots only in the presence of both antibodies, demonstrating a direct interaction between the two proteins.

Altogether, these results indicate that Nectin2 is a direct target of the Ub pathway.

Nectin2 ubiquitination regulates protein stability and surface expression

Since ubiquitinated proteins are mainly subjected to proteasome degradation [30], we investigated whether the Ub pathway controls protein stability.

To this aim we first analyzed by flow cytometry the effect of PYR41 treatment on total Nectin2 levels. In both ARK (Fig. 4A, left panel) and HeLa (Fig. 4A, right panel) cell lines we observed an increase of Nectin2 expression on permeabilized cells. This result was confirmed by confocal microscopy: PYR41 treatment enhances the overall Nectin2 fluorescence intensity (Fig. 4B), supporting the conclusion that ubiquitination contributes to Nectin2 degradation.

Total protein levels of PVR, analyzed as control protein, were not affected by PYR41 treatment (Supporting Information Fig. 1).

To verify that the increased Nectin2 level is not due to a newly synthetized protein pool, we evaluated Nectin2 mRNA level before and after PYR41 treatment. As revealed by real-time-PCR experiments, inhibition of the Ub pathway did not change Nectin2 mRNA levels in both ARK (Fig. 4C, left panel) and HeLa (Fig. 4C, right panel) cells, excluding the contribution of transcriptional regulation or a possible effect of PYR41 on mRNA stability.

To formally demonstrate that Nectin2 is subjected to Ub-dependent degradation, we inhibited proteasome enzymatic activity by means of epoxomicin.

This treatment induced an accumulation of Nectin2 protein level on both ARK (Fig. 5A) and HeLa (Fig. 5B) cells in the absence of protein relocation on cell membrane (Fig. 5C).

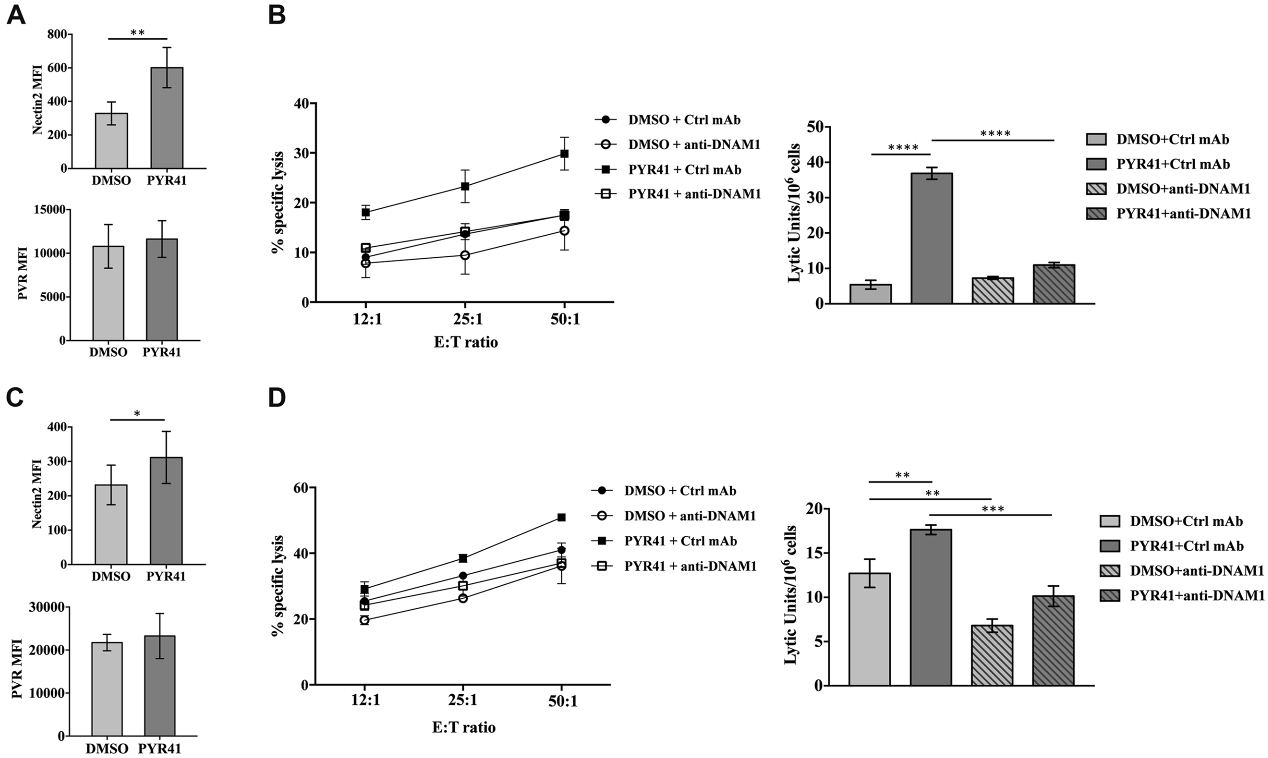

Figure 2. Inhibition of the Ub pathway increases Nectin2 surface expression and tumor cell susceptibility to NK cell-mediated lysis. Inhibition of the Ub pathway on ARK and HeLa cell lines was achieved by means of overnight treatment with 10 μM or 25 μM PYR41, respectively. (A and C) Nectin2 (upper panels) and PVR (lower panels) surface expression was evaluated on ARK (A) and HeLa (C) cell lines by immunofluorescence and FACS analysis. MFI of isotype control Ig was subtracted from Nectin-2 or PVR MFI. Means ± SD ($n = 5$ for ARK and three for HeLa cells). $^{*}p < 0.05$, $^{**}p < 0.01$, by Student's *t*-test. (B and D) Primary cultured NK cells were pretreated for 20 minutes at room temperature with anti-CD226 (DNAM1) neutralizing mAb or with anti-CD56 mAb (Ctrl mAb) and used as effector cells in a 4 hours ^{51}Cr release assay toward ARK (B) or HeLa (D) cells at the indicated effector:target (E:T) ratio. Left panels: percentages of specific lysis, calculated as described in Materials and Methods, are shown as mean ± SD of triplicates from a single representative experiment. Right panels: lytic units, calculated as described in Materials and Methods, from three independent experiments each one performed with $n = 3$ replicates (mean ± SD) are shown. Significant differences were identified by one-way ANOVA with Tukey's post hoc test correction; $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

Thus, the expression of Nectin2 is regulated by Ub-proteasome system (UPS) activity.

A direct link between ER stress response and UPS has been previously reported [36]. Thus, we decided to employ tunicamycin and thaspsigargin to better investigate the contribution of the Ub pathway on Nectin2 expression.

Overnight treatment of ARK and HeLa cells with these stimuli induced an accumulation of protein Nectin2 levels (Fig. 6A and D) and increased its surface expression (Fig. 6B and E) without affecting Nectin2 mRNA levels (Fig. 6C and F).

Interestingly, this increase is accompanied by a reduction of Nectin2 ubiquitination as revealed by Western blotting analysis (Fig. 7).

On primary HFF cells, we found that tunicamycin increases Nectin2 surface expression (data not shown).

Differently from Nectin2, PVR expression does not increase in response to ER stress inducers (Supporting Information Fig. 2).

Altogether, these results support the conclusion that ubiquitination of Nectin2 in addition to promote its degradation also induce protein intracellular retention.

Discussion

Increasing efforts in anti-cancer strategies are aimed to potentiate the ability of cytotoxic immune cells, including CTLs and NK cells, to oppose tumor progression [37].

While CTLs are endowed with the ability to specifically recognize tumor antigens by means of their specific TCR, NK cells do not possess a specific antigen receptor.

Their activation depends on the integration of inhibitory signals transduced by receptors for MHC class I molecules and activating signals induced by recognition of self-molecules nearly absent in normal conditions and upregulated in transformed and virus-infected cells [38]. These molecules trigger activating receptors such as NK group 2D (NKG2D), CD226, and the natural cytotoxicity receptors (NCR), which include NKp46, NKp44, and NKp30 [4, 39–41]. Of note, also some tumor-derived soluble growth factor can increase NK cell anti-tumor responses triggering selected NCRs, as recently described for the NKp44 receptor [42].

Among NK cell activating ligands upregulated in diseased cells, PVR and Nectin2 play a pivotal role in cancer cell clearance since

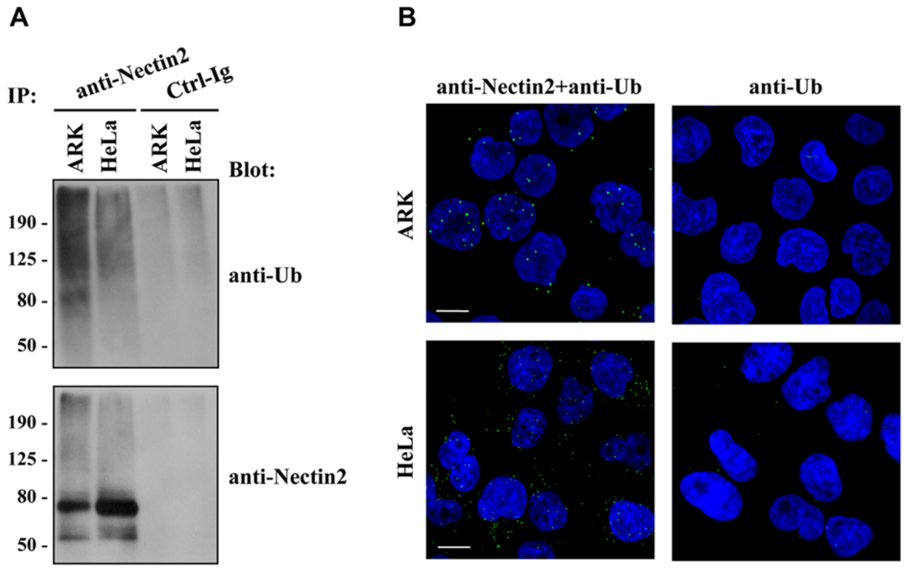

Figure 3. Nectin2 is subjected to Ubiquitination. (A) Cell lysates from ARK or HeLa cells were immunoprecipitated with goat polyclonal anti-Nectin2 or with a goat isotype-matched Ig as a control (Ctrl-Ig). Immunoprecipitated proteins were separated on SDS/PAGE (7.5%), transferred on nitrocellulose filters, and immunoblotted as indicated. One out of three independent experiments is shown. (B) Proximity ligation assay (PLA) was performed on ARK and HeLa cell lines by using polyclonal anti-Ub and/or monoclonal anti-Nectin2 as primary Abs, as indicated. Positive PLA signals are visualized as green fluorescent spots, nuclei are in blue (DAPI). Images are representative of three independent experiments and were acquired using 60X/1.35NA oil immersion objective. Z-projections of 40 slices for ARK cells and 20 slices for HeLa cells are shown. Bar represents 10 μm.

they mediate CD226-dependent costimulation of both NK and $CD8^+$ T cells [9, 10].

Here, we provide the first evidence of a CD226 ligand regulated by the Ub pathway. Our results show that constitutive ubiquitination of Nectin2 prevents ligand expression on cell membrane and affects protein stability, thereby impairing tumor cell recognition by NK cells.

Together with our previous results supporting a role for the SUMO pathway in the regulation of PVR expression, this novel finding demonstrates that different posttranslational modifications cooperate to regulate CD226 ligand membrane expression.

We initially show that Nectin2 intracellular localization is a common feature of both normal and transformed cells. Remarkably, an additional example of Nectin2 intracellular distribution comes from a study on activated T lymphocytes [43].

This expression pattern is compatible with Nectin2 function as adhesion molecule. Indeed, a preferential expression of Nectin protein family on adherent junctions has been reported [44], suggesting that their recruitment on plasma membrane occurs upon cell–cell contacts. In line with this scenario, we have preliminary data demonstrating that a rapid increase in Nectin2 surface expression on tumor cells occurs upon their coculture with HUVEC endothelial cells (data not shown).

On tumor cells, we have also demonstrated that Nectin2 is ubiquitinated and that this modification promotes protein degradation contributing to tumor evasion.

In addition, the Ub pathway is responsible for Nectin2 intracellular retention. Indeed, we found that the reduction of Nectin2 ubiquitination observed in conditions of cellular stress is accompanied by an accumulation of the protein and a concomitant increase of its surface expression.

Of note, inhibition of proteasomal degradation did not alter Nectin2 surface expression suggesting that protein degradation and Nectin2 intracellular retention occurs as two independent events.

Thus, it is likely that the neo-synthetized Nectin2 is ubiquitinated before reaching the plasma membrane and either subjected to degradation or retained intracellularly.

Whether additional Nectin2 posttranslational modifications beside ubiquitination can control cytoplasm-membrane localization remains to be defined.

Regarding other NK activating ligands regulated by Ub modification, only few pieces of evidence are available on NKG2D ligands. The murine ligand, Mult1, has been shown to undergo a constitutive Ub-dependent degradation that can be counteracted by the action of stressing stimuli [45], while the human ligand MICA is ubiquitinated by a viral Ub ligase in Kaposi's sarcoma virus-infected cells [46]. However, upon ubiquitination, MICA does not undergo degradation, but it is retained in intracellular compartments.

Our finding also provided evidence that upon inhibition of Ub pathway, the increased Nectin2 surface expression renders tumor cells more efficiently recognized and lysed by NK cells. Whether this higher susceptibility to NK cell-mediated killing may be also mediated by the upregulation of activating ligands other than Nectin2 is under investigation. At the present, we have excluded the contribution of the other CD226 ligand, PVR, since its surface expression does not increase upon Ub pathway inhibition.

Interestingly, the inhibition of the Ub-proteasome pathway is now under investigation as therapeutic intervention in haematological and solid tumors [47] and it is considered the frontline therapy for MM [48]. In line with our findings, this treatment has been shown to upregulate NK cell activating ligands, including Nectin2 [24, 49].

Several lines of evidence suggest that some viruses may exploit the Ub pathway to downmodulate Nectin2 surface expression in order to escape immune cell recognition.

Upon CMV infection, Nectin2 is retained intracellularly by the viral protein UL141 [26] and degraded through the action of a viral protein, US2, that recruits the cellular ubiquitin ligase TRC8 [26, 50]. In line with these evidences, expression of the gD glycoprotein

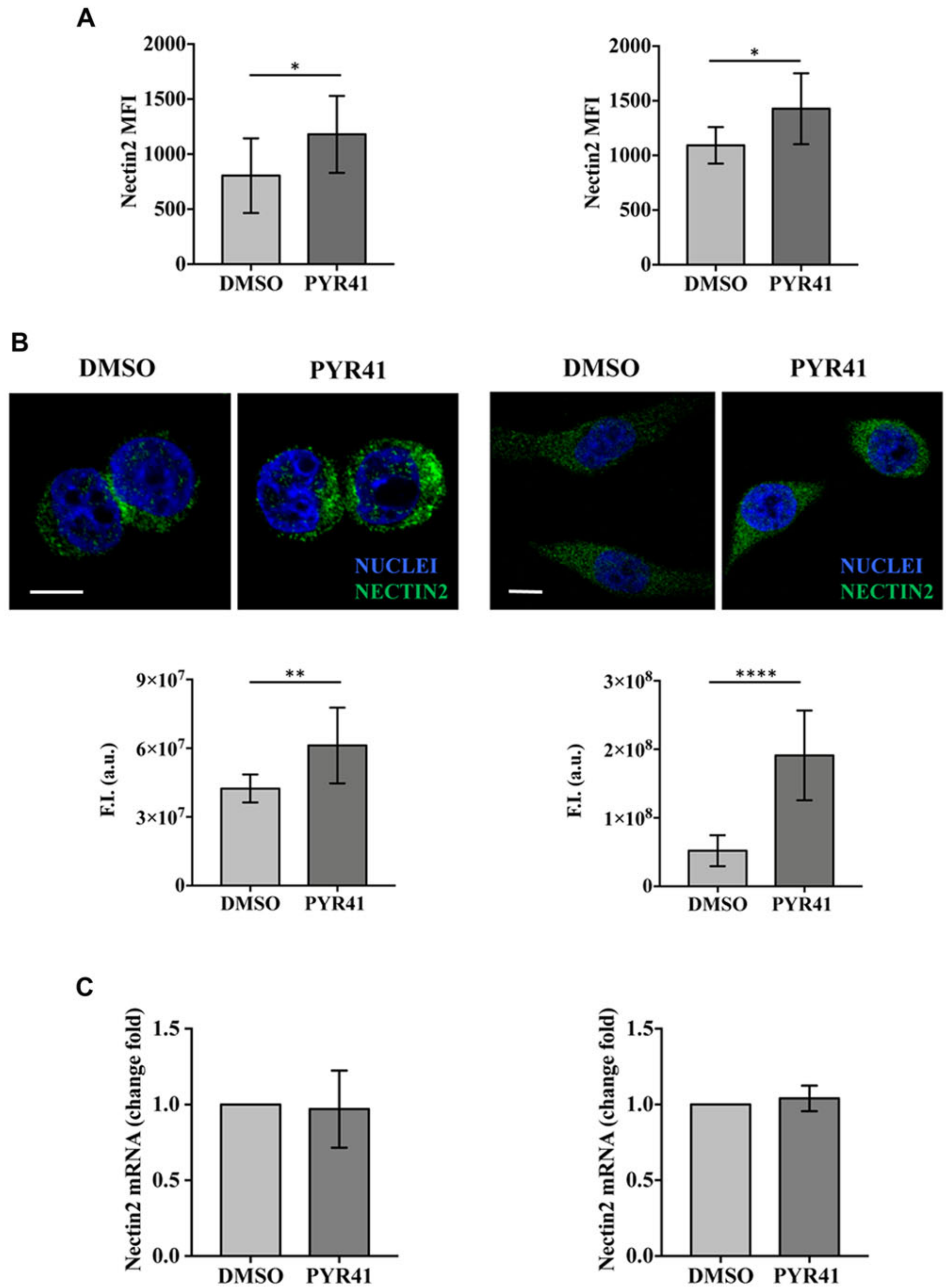

Figure 4. The Ub pathway regulates Nectin2 protein level. Inhibition of the Ub pathway on ARK and HeLa cell lines was achieved as in Fig. 2. (A) Nectin2 total expression levels were evaluated on ARK (left panel) and HeLa (right panel) cells by immunofluorescence and FACS analysis upon fixation and permeabilization using FACSCan to flow cytometer. MFI of isotype control Ig was subtracted from Nectin2 MFI. Means ± SD ($n = 3$ for both ARK and HeLa cells). $^{*}p < 0.05$, by Student's t-test. (B) PYR41 or DMSO-treated ARK (left panels) and HeLa (right panels) cells were stained with anti-Nectin2 mAb followed by Alexa 647-conjugated goat anti-mouse Ab (visualized in green) and counterstained with DAPI (visualized in blue). Images were acquired using 60X/1.35NA oil immersion objective. A single optical slice is shown. Bar represents 10 μm. Green fluorescence intensity (F.I.) was measured with Fiji/ImageJ software in 20 cells randomly acquired from two independent experiments. Bars represent the mean ± SD ($n = 20$). $^{**}p < 0.01$, $^{****}p < 0.0001$, by Student's t-test. (C) Total RNA was used for cDNA first-strand synthesis, and real-time PCR was performed using the ABI Prism 7900 Sequence Detection system. Relative Nectin2 mRNA amount, normalized with GAPDH, was expressed as arbitrary units and referred to DMSO-treated cells, considered as calibrator (the value of the calibrator in each run is set to 1). Means ± SD of three independent experiments (each one with $n = 3$ replicates) are shown.

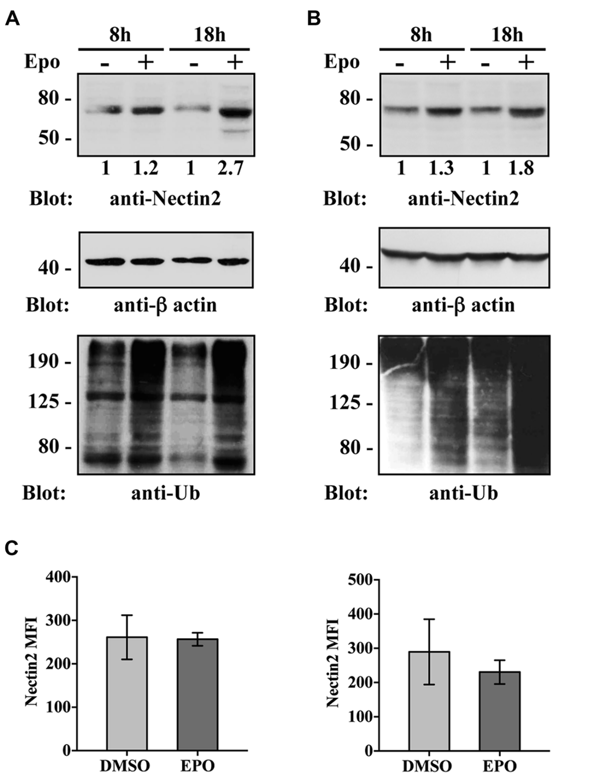

Figure 5. Nectin2 undergoes proteasome degradation. (A and B) Proteasome inhibition on ARK (A) and HeLa (B) was achieved by treatment with 2 μM Epoxomicin (Epo) for the indicated lengths of time. Cells were lysed, and total cell lysates were separated on SDS-PAGE (7.5%) and transferred on a nitrocellulose filters. Western blotting was performed with goat polyclonal anti-Nectin2 and monoclonal anti-β actin Abs to verify equal protein loading. The relative Nectin2 amount was calculated by normalizing to the level of β-actin and expressed as fold change respect to untreated samples (arbitrarily set to 1). The treatment efficiency was verified by performing anti-Ub blot. Results shown are representative of three independent experiments. (C) Nectin2 surface expression was evaluated on ARK (left panel) and HeLa (right panel) cell lines by immunofluorescence and FACS analysis. MFI of isotype control Ig was subtracted from Nectin-2 MFI. Means ± SD (n = 3 for both ARK and HeLa cells).

of pseudorabies virus (PRV) and Herpes Simplex Virus 2 (HSV-2) leads to degradation of Nectin2 reducing its cell surface levels and decreasing NK cell-mediated lysis [51].

In conclusion, our results support a model in which the Ub pathway negatively regulates Nectin2 surface expression by targeting the protein for degradation but also by promoting Nectin2 intracellular retention.

In healthy cells, these modifications may act to prevent ligand membrane expression and a potential aberrant NK cell activation, while in viral-infected or transformed cells may represent a mechanism to evade NK-cell surveillance. Since CD226 is also expressed on other immune cells, including $CD8^+$ T cells, the biological consequences of a rapid kind of Nectin2 and PVR regulation may not be limited to NK cells.

In addition to shed new light on the regulation of Nectin and Nectin-like protein expression, our finding suggests that the Ub and Ub-like CD226 ligand modifications may represent a potential target to improve immune cell responsiveness to viruses and cancer.

Materials and methods

Cell lines and drug treatments

The human MM cell line ARK (kindly provided by Prof. P. Trivedi, "Sapienza" University of Rome), the human breast cancer cell line (MDA-MB-231), the human cervical cancer cell line HeLa, the human leukemia cell line (K562) were periodically tested for mycoplasma contamination by EZ-PCR Mycoplasma Test Kit (Biological Industries). All cell lines were kept in culture for less than two consecutive months.

ARK, MDA-MB-231, K562 cell lines were maintained at 37°C in 5% CO_2 in RPMI 1640 (Euroclone) supplemented with 10% foetal calf serum (FCS); HeLa cell line was maintained in DMEM (Euroclone) supplemented with 10% FCS.

The HUVEC were purchased by American Type Culture Collection (ATCC) and cultured between passages 2 and 5 in EGM-2 Bulletkit medium (Lonza) supplemented with 2% fetal bovine serum (FBS).

The HFF were purchased by ATCC and cultured between passages 2 and 10 in DMEM supplemented with 10% FCS.

Primary cultured human NK cells were obtained from 10-day cocultures of PBMCs with the irradiated EBV-transformed B-cell line RPMI 8866 at 37°C in 5% CO_2 in the absence of IL-2, as previously described [52].

For experiments in which the Ub pathway was inhibited, MM cells were cultured overnight at 37°C in 5% CO_2 at the concentration of 2×10^6 cells/mL with different doses of PYR41 (Calbiochem) or equal amounts of DMSO. A dose–response assay was performed to identify the amount that did not affect cell viability and the dose of 10 and 25 μM was chosen for ARK and HeLa cells, respectively.

For experiments requiring proteasome inhibition, cells were pretreated at the concentration of 2×10^6 cells/mL for 8 or 18 hours with 2 μM Epoxomicin (Enzo Lifesciences).

For experiments requiring ER stress inhibitors, cells were pretreated for 18 hours with 2 μM Tunicamycin (SIGMA Aldrich) or with 3 μM Thapsigargin (SIGMA Aldrich).

Immunofluorescence and FACS analysis

Surface Nectin2 and PVR expression was evaluated by means of APC-conjugated anti-Nectin2 (R&D Systems, clone # 610603) and PE-conjugated anti-PVR (BioLegend, clone # SKII.4) monoclonal antibodies (mAbs), respectively.

To evaluate total cellular CD226 ligands, MM cells were fixed with 2% paraformaldehyde (Sigma Aldrich, 158127) for 20 minutes at room temperature, permeabilized with 0.1% saponin (Sigma Aldrich, S7900) for 30 minutes at room temperature,

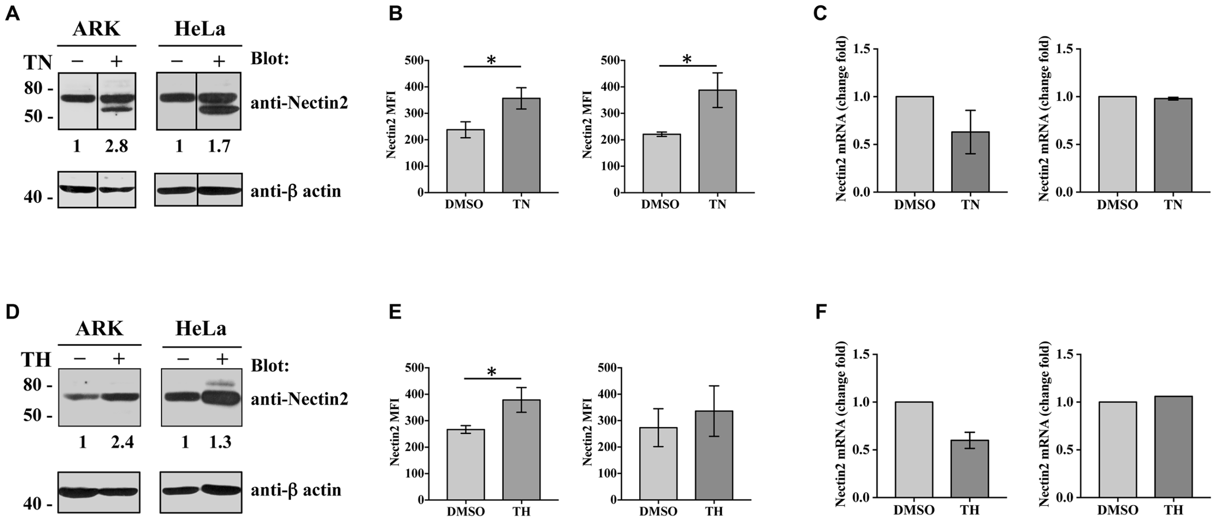

Figure 6. Treatment with ER stress inducers increases Nectin2 protein levels. ARK and HeLa cell lines were treated for 18 hours with 2 μM Tunicamycin (TN) (A–C) or with 3 μM Thapsigargin (TH) (D–F). (A and D) ARK (left panels) and HeLa (right panels) cell lysates were separated on SDS-PAGE (10%), and transferred on a nitrocellulose filters. Western blotting was performed with goat polyclonal anti-Nectin2 and monoclonal anti-β actin Abs to verify equal protein loading. The relative Nectin2 amount was calculated by normalizing to the level of β-actin and expressed as fold change respect to untreated samples (arbitrarily set to 1). The data shown are representative of three independent experiments. (B and E) Nectin2 surface expression was evaluated on ARK (left panels) and HeLa (right panels) cells by immunofluorescence and FACS analysis. MFI of isotype control Ig was subtracted from Nectin2 MFI. Data were presented as means ± SD ($n = 3$ for both ARK and HeLa cells). $^*p < 0.05$, determined by paired Student's *t*-test. (C and F) Total RNA from ARK (left panels) and HeLa (right panels) was used for cDNA first-strand synthesis, and real-time PCR was performed using the ABI Prism 7900 Sequence Detection system. Relative Nectin2 mRNA amount, normalized with GAPDH, was expressed as arbitrary units and referred to DMSO-treated cells, considered as calibrator (the value of the calibrator in each run is set to 1). Means ± SD of three independent experiments (each one with $n = 3$ replicates) are shown.

and washed with PBS 0.5% BSA before the staining. Samples were acquired using a FACSCanto (BD Biosciences) and analyzed using FlowJo software (Ashland, OR) and cells gated based on forward scatter plot versus side scatter plot (Supporting Information Fig. 3).

Confocal microscopy and proximity ligation assay

ARK cells were plated on poly-L-lysine-coated multichamber slides (LabTek, Thermo Scientific) and let adhere for 30 minutes at 37°C. Cells were then fixed and permeabilized, as previously described [53]. HeLa cells were left to grow overnight on gelatine-coated multichamber slides before fixation and permeabilization.

In experiments in which Nectin2 and PVR expression was analyzed, cells were stained with mouse anti-Nectin2 (BD Biosciences, clone # R2.525) or mouse anti-PVR (ThermoFisher Scientific, clone # D171) mAb followed by the AlexaFluor 647-conjugated goat anti-mouse IgG (Life Technologies). After extensive washing, cells were counterstained with DAPI (Life Technologies) and coverslips were mounted using SlowFade gold reagent (Life Technologies).

Proximity ligation assay (PLA) was performed using Duolink PLA in situ Green Starter Kit (Sigma Aldrich, Mouse/Rabbit) according to manufacturer's instructions, as previously described [29]. Mouse monoclonal anti-Nectin2 (BD Biosciences, clone # R2.525) and rabbit polyclonal anti-Ub (Enzo Lifesciences) were used as primary Abs.

Images were acquired at room temperature using IX83 FV1200 MPE laser-scanning confocal microscope with a 60 × 1.35 NA UPlanSAPO oil immersion objective (all from Olympus), as previously described [53]. Images were processed with Fiji ImageJ software. Where indicated, image stacks were acquired.

RNA isolation, RT-PCR, and real-time PCR

Total RNA was extracted using TRIzol (Life Technologies), according to manufacturer's instructions, and measured as previously described [29]. One microgram of total RNA was used for cDNA first strand synthesis according to the manufacturer's protocol for Moloney murine leukemia virus reverse transcriptase (Promega) and real-time PCR was performed using the ABI Prism 7900 Sequence Detection system (Applied Biosystems), as previously described [29]. Briefly, cDNAs were amplified in triplicate with primers for Nectin2 (Hs01071562_m1) and GAPDH (Hs99999905_m1) both conjugated with fluorochrome FAM (Applied Biosystems). The level of ligand expression was measured using threshold cycle (Ct). The Ct was obtained by subtracting the Ct value of the gene of interest (Nectin2) from that of housekeeping gene (GAPDH). Ct of the untreated sample was used as the calibrator and the fold change was calculated as $2^{-\Delta\Delta Ct}$, where ΔΔCt is the difference between the Ct of the sample and the Ct of the calibrator. The analysis was performed using SDS version 2.2 software (Applied Biosystems).

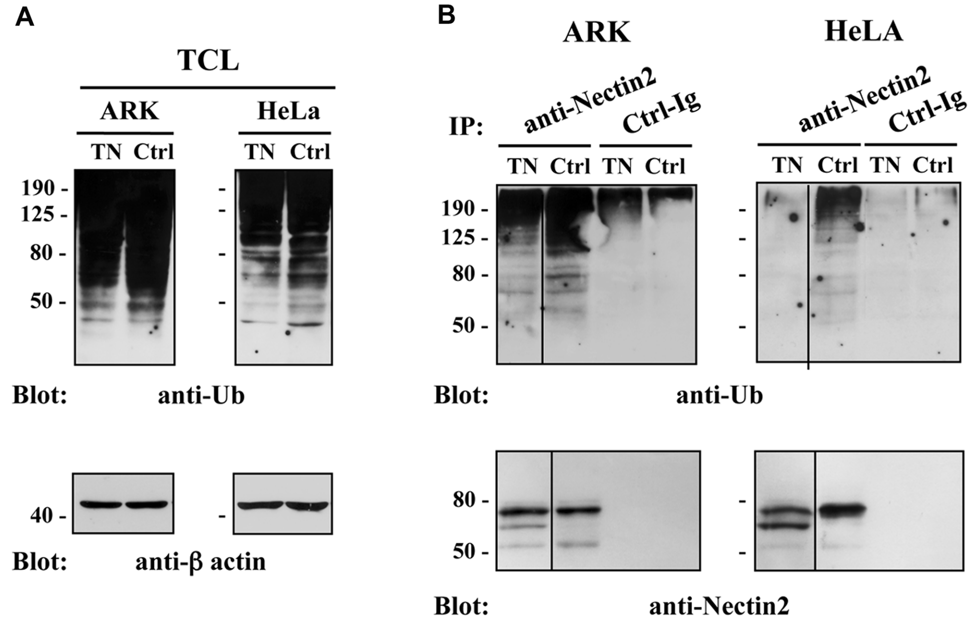

Figure 7. Tunicamycin treatment affects Nectin2 ubiquitination. ARK and HeLa cell lines were treated for 18 hours with 2 μM Tunicamycin (TN). (A) Total cell lysates were separated on SDS-PAGE (10%) and transferred on a nitrocellulose filter. Western blotting was performed with anti-Ub and anti-β actin mAbs to verify equal protein loading. (B) Cell lysates were immunoprecipitated with goat polyclonal anti-Nectin2 Ab or with a goat isotype-matched Ig as a control (Ctrl-Ig). Immunoprecipitated proteins were separated on SDS/PAGE (10%), transferred on nitrocellulose filters, and immunoblotted as indicated. One out of three independent experiments is shown.

Immunoprecipitation and Western blot analysis

Cells were lysed in a buffer containing 1% Triton X-100, 0.1% SDS, 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.5% sodium deoxycholate, 1 mM EGTA, 1 mM EDTA, 5 mM $MgCl_2$, 1 mM PMSF, 1 mM Na_3VO_4, 5 mM NaF pH 8, 20 mM N-ethyl-maleimide, 10 μM of aprotinin, and 5 μM leupeptin, incubated 30 minutes on ice and then centrifuged at 13 000 × g for 30 minutes at 4°C and the supernatant was collected as whole-cell extract. Bio-Rad Protein Assay was used to determine protein concentration.

Immunoprecipitation was performed as previously described [54] using goat polyclonal anti-Nectin2 (R&D Systems) or control goat IgG (Santa Cruz Biotechnology). Immunoprecipitates or total cell lysates were resolved by SDS-PAGE, proteins were then electro-blotted onto nitrocellulose membranes (GE Healthcare), and Western blotting was performed as previously described [54] with goat polyclonal anti-Nectin2 (R&D Systems), monoclonal anti-β actin (Sigma Aldrich, clone # AC-15), or monoclonal anti-Ub (Enzo Lifesciences, clone # FK2) Ab.

Fiji Image J software was used to perform densitometric analysis.

Cytotoxicity assay

NK cells were pretreated with anti-CD226 (Serotec, clone # DX11) or anti-CD56 (Serotec, clone # NCAM16.2) mAb at 1 μg/10^6 cells for 20 minutes at room temperature and employed as effector cells in cytotoxicity assays using as cell targets ARK or HeLa cells treated or not with PYR41.

The ^{51}Cr-release assay (4 hours) was used to measure cytotoxic activity against ARK or HeLa target cells, as previously described [55]. Maximum release was assessed by incubating ^{51}Cr-labeled target cells with 2.5% SDS, while spontaneous release was evaluated by incubating the target cells alone in culture medium. The percentage of specific lysis was determined as follows: ((mean cpm experimental release – mean cpm spontaneous release)/(mean cpm maximal release – mean cpm spontaneous release)) × 100. Lytic units for 10^6 effector cells were calculated as follows: 10^6/(number of target cells × X), where X is the E:T ratio resulting in 15% specific lysis for ARK cells or 30% specific lysis for HeLa cells.

Statistical evaluation

Statistical significance between two groups was determined by performing two-tailed, paired Student's t-test using Prism version 7.2 (GraphPad Software). For analysis of differences among three or more groups, one-way ANOVA with Tukey correction was performed.

Graphs show mean values, and all error bars represent the SD.

Acknowledgments: We thank B. Milana for her technical assistance and the CLNS imaging facility of the Istituto Italiano di Tecnologia (Rome, Italy) where the fluorescent images were collected.

This work has been supported by the Italian Association for Cancer Research (AIRC) Investigator Grant number 16014, AIRC 5xmille under Grant number 9962, and Italian Ministry of Education, Universities and Research—Dipartimenti di Eccellenza—L. 232/2016.

R.M. performed the experiments, analyzed results, and contributed to design research and to write the manuscript; N.D.M. performed experiments, analyzed results, and contributed to write the manuscript, B.Z. and M.L. performed experiments, and analyzed results; C.F. and M.C. critically reviewed the manuscript; A.S. contributed to design research; R.P. contributed to design research and write the manuscript.

Conflict of interest: The authors declare no commercial or financial conflicts of interest.

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Abbreviations: **DNAM1**: DNAX-associated molecule1 · **HFF**: human foreskin fibroblast · **MM**: multiple myeloma · **NCR**: natural cytotoxicity receptor · **PLA**: proximity ligation assay · **PVR**: poliovirus receptor · **TH**: thapsigargin · **TN**: tunicamycin · **Ub**: ubiquitin

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The peer review history for this article is available at https://publons.com/publon/10.1002/eji.201847848

Received: 4/8/2018
Revised: 4/2/2019
Accepted: 14/3/2019
Accepted article online: 19/3/2019

