Immunobiology 226 (2021) 152070

Contents lists available at ScienceDirect

Immunobiology

journal homepage: www.elsevier.com/locate/imbio

# Selective HSP90β inhibition results in TNF and TRAIL mediated HIF1α degradation

A.L. Heck [a], S. Mishra [b], T. Prenzel [c], L. Feulner [c], E. Achhammer [c], V. Särchen [a], B.S.J. Blagg [b], W. Schneider-Brachert [c], S. Schütze [a], J. Fritsch [a,c,*]

[a] Institute of Immunology, Christian-Albrechts-University of Kiel, 24105 Kiel, Germany
[b] Department of Chemistry and Biochemistry, The University of Notre Dame, Notre Dame, IN 46556, United States
[c] Department of Infection Prevention and Infectious Diseases, University of Regensburg, 93053 Regensburg, Germany

ARTICLE INFO

*Keywords:*
TNF
TRAIL
HSP90
Apoptosis

ABSTRACT

Signaling via TNF-R1 mediates pleiotropic biological outcomes ranging from inflammation and proliferation to cell death. Previous reports demonstrated that pro-survival signaling emanates from membrane resident TNF-R1 complexes (complex I) while only internalized TNF-R1 complexes are capable for DISC formation (complex II) and thus, apoptosis induction. Internalized TNF-R1 containing endosomes undergo intracellular maturation towards lysosomes, resulting in activation and release of Cathepsin D (CtsD) into the cytoplasm. We recently revealed HSP90 as target for proteolytic cleavage by CtsD, resulting in cell death amplification.

In this study, we show that extrinsic cell death activation via TNF or TRAIL results in HSP90β degradation. Co-incubation of cells with either TNF or TRAIL in combination with the HSP90β inhibitor KUNB105 but not HSP90α selective inhibition promotes apoptosis induction. In an attempt to reveal further downstream targets of combined TNF-R1 or TRAIL-R1/-R2 activation with HSP90β inhibition, we identify HIF1α and validate its ligand: inhibitor triggered degradation.

Together, these findings suggest that selective inhibition of HSP90 isoforms together with death ligand stimulation may provide novel strategies for therapy of inflammatory diseases or cancer, in future.

## 1. Introduction

A central topic of current tumor research is to unravel the mechanisms of immune evasion of tumors against the self-defense of the immune system. It is well known that extrinsic cell death of tumor cells is regulated via the death receptors *tumor necrosis factor receptor 1* (TNF-R1) or *TNF-related apoptosis inducing ligand receptor 1* or *2* (TRAIL-R1/R2). Their regulation is strongly tumor- and cell type dependent. Both, TNF and TRAIL induce their biological activity via binding to their respective receptors of the family of death receptors TNF-R1, TRAIL-R1 and -R2 and can transmit both, cell death (apoptosis and necroptosis) as well as inflammatory and proliferative signals (Schütze et al., 2008). To date there is no conclusive explanation as to how these diametrically opposed biological activities are elicited via the binding of the same ligand to its respective receptor. Our group and others have shown that signaling of death receptors depends on their subcellular localization. i.e. membrane resident TNF-R1 signals pro-survival, while apoptosis signaling depends on its internalization and signaling from TNF-receptosomes (Mahul-Mellier et al., 2008; Chhibber-Goel et al., 2016; Holdbrooks et al., 2018; Ali et al., 2013; Fritsch et al., 2014; Schneider-Brachert et al., 2006, 2004). The mechanism by which TRAIL induces signaling and receptor internalization remains to be elucidated, as different cell lines exhibit different response towards ligand binding (Zhang et al., 2009; Akazawa et al., 2009; Mazurek et al., 2012; Austin et al., 2006; Kohlhaas et al., 2007; Sosna et al., 2016).

We recently identified the molecular chaperone HSP90 as a novel proteolytic substrate of the lysosomal aspartic protease Cathepsin D (Fritsch et al., 2016). Inhibition of HSP90 with the inhibitor 17AAG resulted in amplification of both, TNF and TRAIL induced apoptotic response a cell type dependent manner (Fritsch et al., 2016). The HSP90 family comprises four members: HSP90aa1 (HSP90α1), HSP90ab1 (HSP90β), HSP90b1 (GRP94) and HSP90L (TRAP-1) (Kampinga et al., 2009). While HSP90α1 is the inducible isoform, HSP90β is the constitutively expressed variant in the cytoplasm. HSP90b1 is ER localized and

* Corresponding author at: Department of Infection Prevention and Infectious Diseases, University of Regensburg, Franz-Josef-Strauss-Allee 11, 93053 Regensburg, Germany.
*E-mail address:* Juergen.Fritsch@ukr.de (J. Fritsch).

https://doi.org/10.1016/j.imbio.2021.152070
Received 3 November 2020; Received in revised form 23 December 2020; Accepted 31 January 2021
Available online 5 February 2021

HSP90L is localized in mitochondria. The structure of all mammalian HSP90 isoforms is similar. The C-terminal part is required for the formation of HSP90 homodimers and contains a conserved EEVD motive, which is required for the recruitment of co-chaperones. More than 20 proteins have been shown to regulate HSP90 function as co-chaperones. This interaction is required for controlling the ATPase activity of HSP90 as well as for recruitment of other proteins (Verma et al., 2016). The middle region of HSP90 functions as linker and provides a high affinity interaction platform for co-chaperones and client proteins. Inhibition of the HSP90 ATPase activity interferes with the correct folding of various client proteins. Consequently, client proteins undergo destabilization, ubiquitination and subsequent proteasomal degradation (Verma et al., 2016; Calderwood and Gong, 2016; Pandey et al., 2016).

HSP90 has been associated with the growth and propagation of various tumor types, making it a veritable target for anti-tumor intervention. As HSP90 is essential for the stabilization and function of various oncogenes, inhibition of HSP90 using small molecules presents an exciting opportunity to treat cancer by regulating multiple downstream effectors. For this reason, various compounds have been reported that target HSP90. Many of these have promising anti-tumor activity and are currently evaluated in clinical trials (Verma et al., 2016; Pandey et al., 2016; Garg et al., 2016; Khandelwal et al., 2016). Combination therapies of HSP90 inhibitors together with other chemotherapeutic agents are emerging as promising approaches to overcome resistance towards single agent therapies. Death ligands of the TNF family, especially TRAIL, are promising targets for the treatment of tumors, in combination therapy (Prasad et al., 2014; Lim et al., 2015; Trivedi and Mishra, 2015; Sanchez et al., 2020).

Based on our recent observations, we here show that stimulation of cells with TNF or TRAIL leads to selective HSP90β degradation. Inhibition of HSP90β using novel isoform specific inhibitors boosts both TNF and TRAIL mediated apoptosis and induces degradation of the pro survival transcription *factor hypoxia induced factor 1 alpha* (HIF1α).

## 2. Results

### 2.1. TNF and TRAIL treatment selectively induces HSP90β cleavage

To extend our recent findings of HSP90 cleavage in response to different death ligands (Fritsch et al., 2016), we first aimed to clarify which HSP90 isoform is involved. To this, U937 cells were treated with TNF or TRAIL for 4 or 8 h. We focused on U937 cells as moderate levels of apoptosis can be triggered using TNF only, whereas most other cell lines require additional sensitization by cycloheximide (CHX) to induce apoptosis. We found that only HSP90β is cleaved while HSP90α is not cleaved (Fig. 1A and B). HSP90β cleavage is strongest upon TRAIL

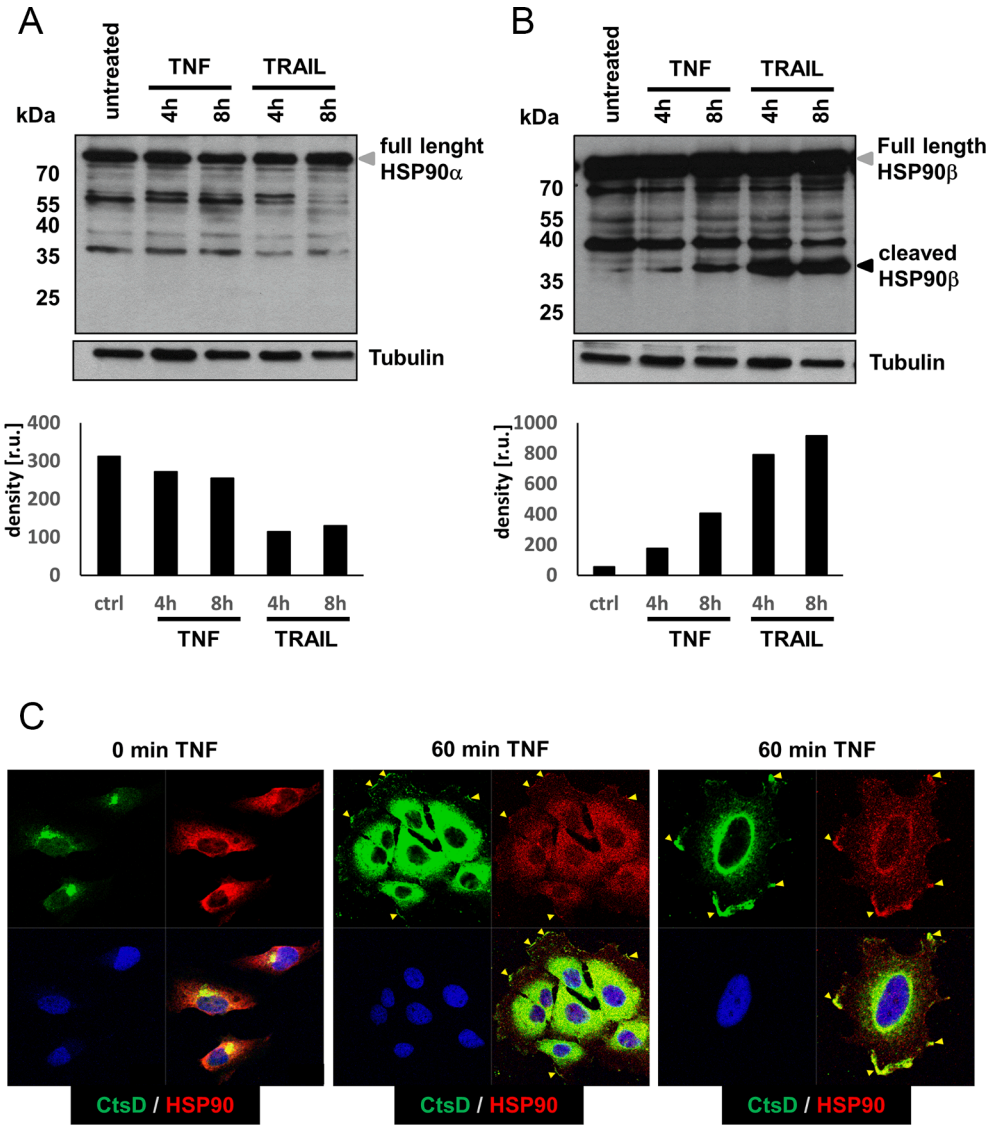



**Fig. 1.** TNF and TRAIL treatment induces selective HSP90β cleavage. U937 cells were treated for 4 h or 8 h with death ligands and compared to untreated lysate. A) WB was probed for HSP90α. B) WB was probed for HSP90β. Arrows indicate full length (grey) or cleaved HSP90 (black). Tubulin serves as loading control. Densitometric analysis of the bands is shown below the respective WB (r.u = relative units). One representative experiment of n = 3 is shown. C) Fluorescence micrograph of HeLa cells without (left panel) and with 60 min TNF stimulation (middle and right panel). CtsD is stained in green, HSP90 in red, nuclei are stained in blue. Partial co-localizaton at the plasma membrane is indicated by yellow arrow heads in the middle and right panel. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

treatment.

Interestingly, neither in this study, nor in the previous one, we observed a strong decrease of the full length HSP90β band, concomitant with the appearance of the cleavage product (Fritsch et al., 2016). Assuming that HSP90β is degraded selectively in a distinct subcellular location, we performed fluorescence imaging to investigate where HSP90β and CtsD co-localize. Fig. 1C shows partial co-localization of CtsD and HSP90 after stimulating cells for 60 min with TNF.

To investigate the time of onset of HSP90β cleavage in response to TNF and TRAIL, U937 cells were incubated with the ligands from 0 min to 4 h. For TNF, HSP90β fragmentation occurs at 3 h (Fig. 2A), while TRAIL induced cleavage is already visible at 2 h (Fig. 2B).

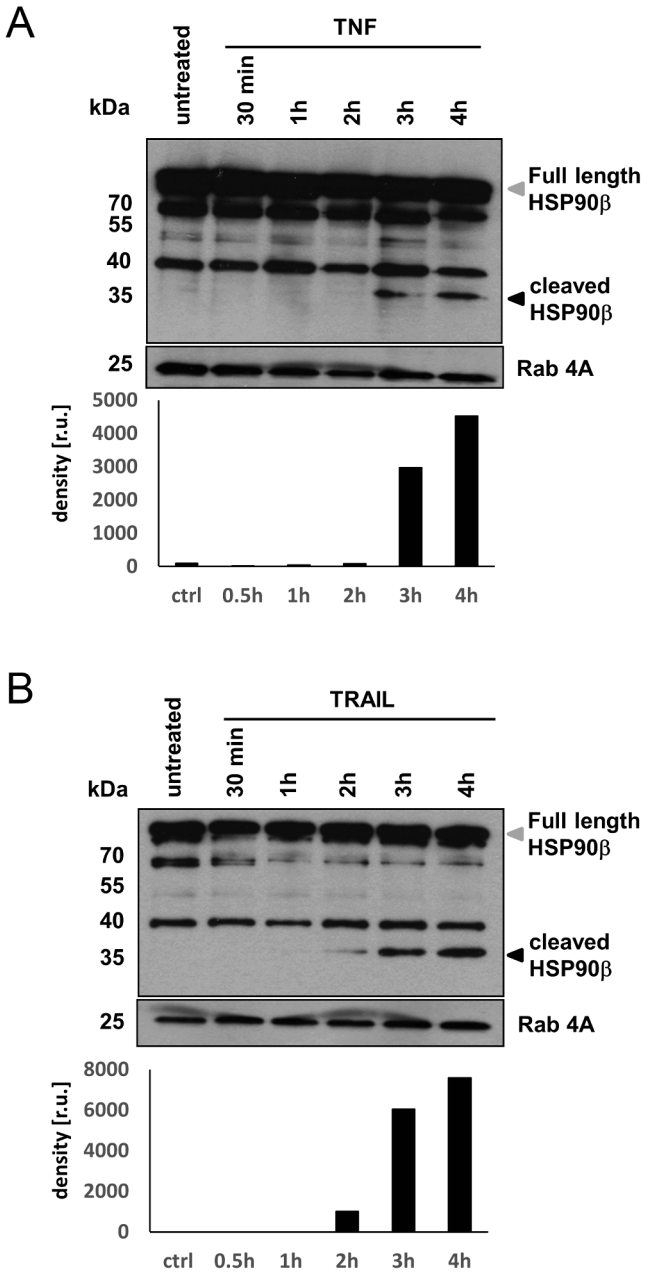



**Fig. 2.** Onset of TNF and TRAIL induced HSP90β cleavage. U937 cells were treated for 30 min up to 4 h with TNF or TRAIL and compared to untreated lysate. A) Lysates after TNF treatment. WB was probed for HSP90β. B) Lysates after TRAIL treatment. WB was probed for HSP90β. Arrows indicate full length (grey) or cleaved HSP90β (black). Rab4 serves as loading control. Densitometric analysis of the bands is shown below the respective WB (r.u = relative units). One representative experiment of n = 3 is shown.

### 2.2. Selective inhibition of HSP90β increases TNF and TRAIL induced apoptosis

Our previous report showed that HSP90 inhibition enhances ligand-mediated apoptosis (Fritsch et al., 2016). To evaluate the biological effect of isoform-specific HSP90 inhibitors we first performed apoptosis assays, measuring nuclear DNA fragmentation, to define their toxicity threshold (Fig. 3A). Compound KUNA110 is alpha selective, compound KUNB105 is beta selective and the third was a pan-HSP90 inhibitor. Non toxic concentrations were used for further experiments. Fig. 3B shows that low concentration of KUNB105 (750 nM), KUNA110 (2.5 μM) or pan-HSP90 inhibitors (1.5 μM) is not toxic by overnight incubation (light grey bars). Overnight incubation with TNF triggers apoptosis, which can be increased by co-incubation with a KUNB105 or the pan-HSP90 inhibitor (dark grey bars). Overnight incubation with TRAIL +/− inhibitors triggers a similar response (black bars). Inhibition of HSP90α does not affect cell death. Interestingly, we observed no increase in cell death when using the HSP90β selective inhibitor Gambogic acid and its derivative DAP-19 (not shown).

Similar effects were observed using Annexin V/7-AAD staining to monitor cell death (Fig. 4A and B): TNF alone induces marginal cell death whereas co-incubation with KUNB105 or the pan-HSP90 inhibitor has the same effect as CHX co-incubation. Co-incubation of TRAIL with either KUNB105 or the pan-HSP90 inhibitor increased cell death, too.

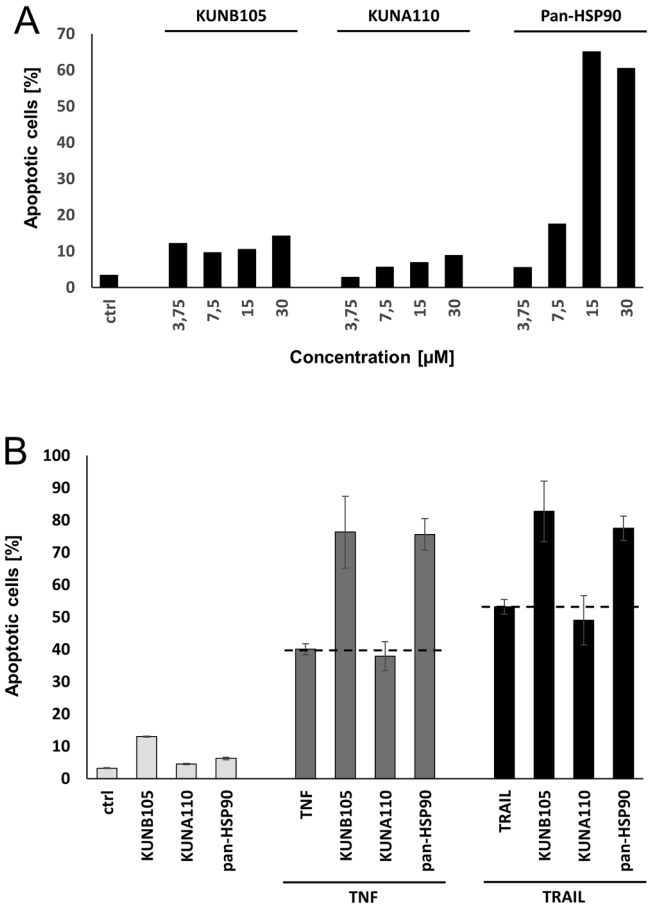



**Fig. 3.** Selective inhibition of HSP90β increases TNF and TRAIL induced HSP90β cleavage. Apoptosis induction in U937 cells was quantified by ImageStream. A) Non toxic concentrations were determined for the inhibitors KUNB105; KUNA110; and the pan-HSP90 inhibitor 17AAG. B) Light grey bars indicate untreated cells or cells treated with non toxic inhibitor concentrations (KUNB105: 750 nM; KUNA110: 2.5 μM; pan: 1.5 μM). Medium grey bars indicate (co-)treatment with TNF. Black bars indicate (co-)treatment with TRAIL. One representative experiment of n = 3 is shown.

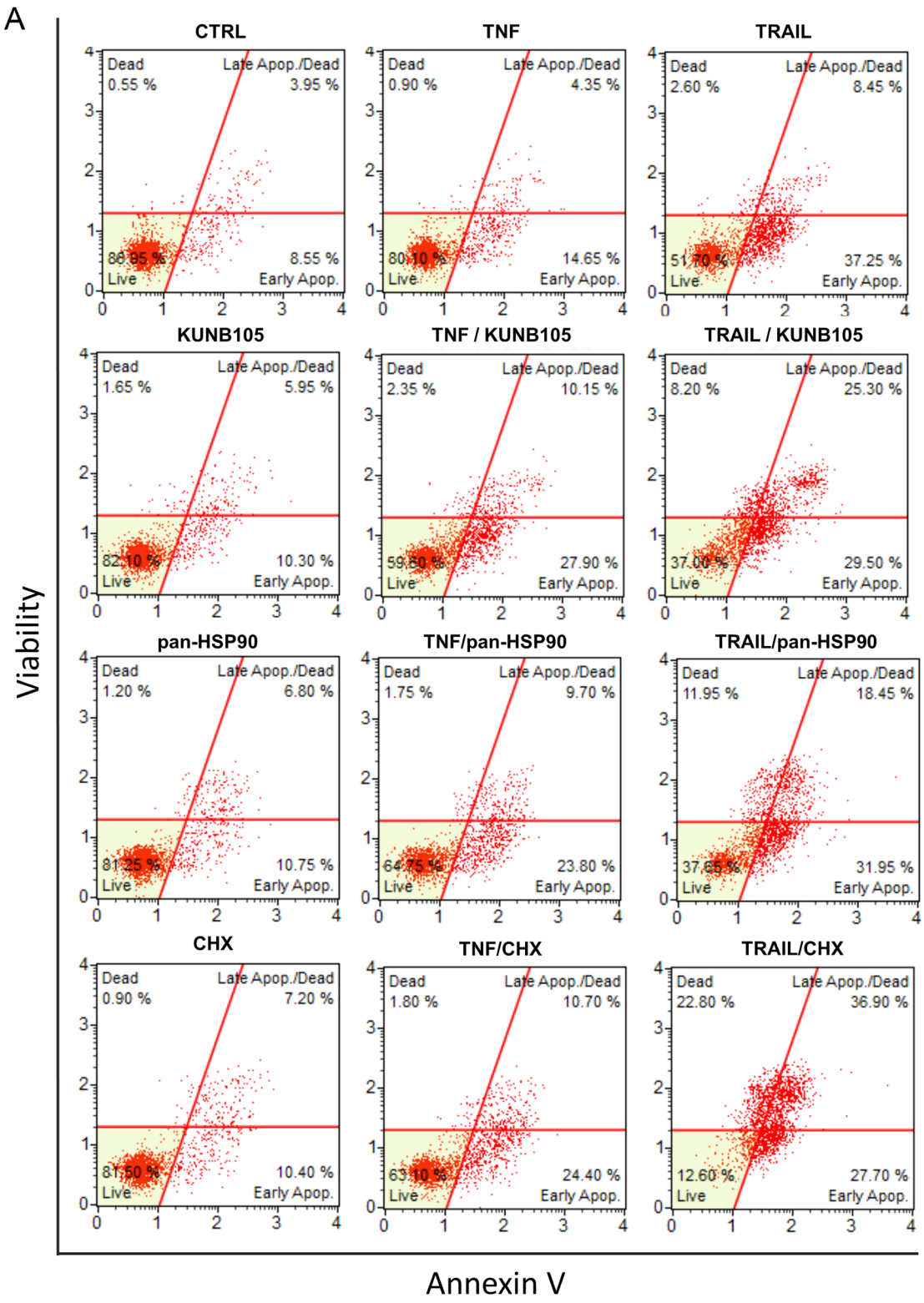



**Fig. 4.** Cell death analysis comparting TNF and TRAIL incubation with HSP90 inhibitors and CHX. Apoptosis induction was monitored using the Guava MUSE Annexin V/7-AAD assay. A) Living cells are shown in the lower left part of the respective plots. Dying/dead cells shift to the right and upper part of the plot. B) Shows the total apoptotic values in %. One representative experiment of n > 3 is shown. C) Activation (cleavage), in response to 6 h co-incubation with TNF or TRAIL and KUNB105, of the caspases 8 and 9 as well as PARP was analyzed by WB. Tubulin served as loading control. Densitometric analysis of the bands is shown below the WB panels (r.u = relative units).

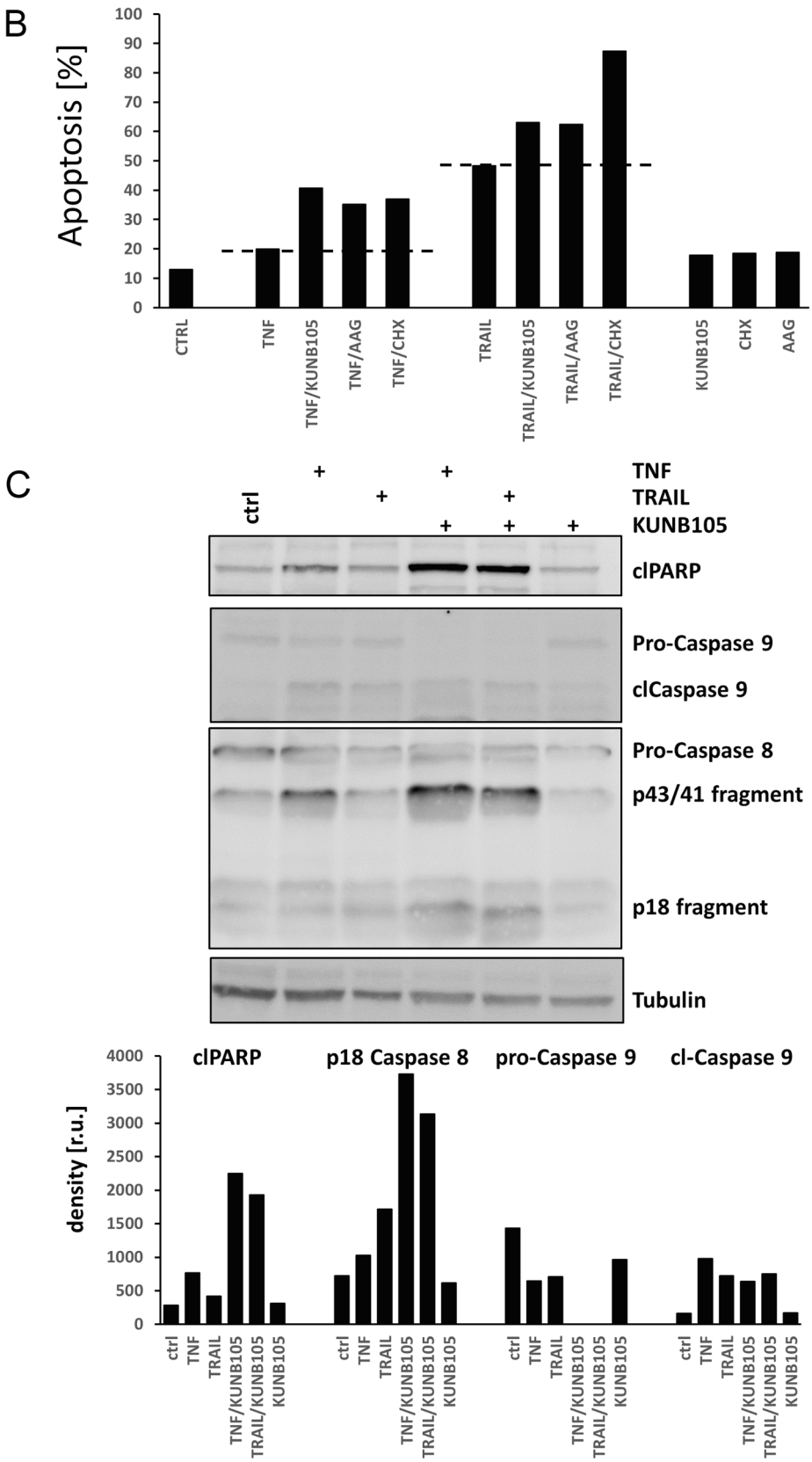



**Fig. 4.** (*continued*).

However, the effect was less pronounced as for CHX co-incubation.

In our previous publication, we showed that panHSP90 inhibition resulted in enhanced Bid cleavage and caspase9 activation in response to TNF stimulation (Fritsch et al., 2016). Activation of caspase9 downstream of TNF-R1 activation requires TNF-R1 internalization and intracellular maturation of TNF-receptosomes to the lysosomal

compartment and apoptosis amplification my lysosomal and MOMP (Schneider-Brachert et al., 2004; Edelmann et al., 2011; Heinrich et al., 2004). For TRAIL signaling this is not as clear. We here investigated which caspases are activated upon 6 h co-incubation TNF or TRAIL and KUNB105. Fig. 4C shows that both, caspase 8 and 9 are activated in response to the stimulus.

### 2.3. Selective inhibition of HSP90β increases TNF and TRAIL induced HSP90β cleavage

To investigate if TNF/inhibitor co-treatment affects HSP90β cleavage, U937 cells were incubated with TNF and with or without additional inhibitor (Fig. 5A and B). For both ligands, HSP90β cleavage is enhanced in the presence of the HSP90β-selective inhibitor.

### 2.4. NF-κB signaling via TNF or TRAIL is not affected by selective HSP90β inhibition

Both TNF and TRAIL can activate non-death signaling via NF-κB. For TNF, this represents even the predominant signaling cascade in most cell lines. Fig. 6 shows degradation of IκB as readout for NF-κB activation. The response was quantified by western blot in both TNF and TRAIL treated cells with and without inhibition of HSP90β.

### 2.5. Selective inhibition of HSP90β results in HIF1α degradation

As HSP90 is known to exert its anti-apoptotic activity by stabilizing various protective proteins, we aimed to identify downstream effectors/HSP90 clients. We performed Proteome Profiler array analysis (Human Cell Stress and Human Apoptosis Array) from untreated, KUNB105, TNF and KUNB105/TNF treated U937 cells (Fig. 7A and B). Fig. 7A shows decreasing intensity for HIF1α staining (red box) in both arrays upon overnight co-incubation with inhibitor and TNF. Fig. 7B shows the densitometric quantification analysis of the respective spots.

These results indicate a functional link between HSP90β and the anti-apoptotic protein HIF1α within death ligand mediated apoptosis regulation. As HIF1α is known to interact with and its stability depends on active HSP90 we further aimed to validate these findings (Liu and Semenza, 2007). Therefore, U937 cells were incubated for 4 or 20 h at different conditions (Fig. 7C). We observed diminished HIF1α signals in cells incubated overnight in the presence of TNF and HSP90β inhibitor KUNB105. This cleavage could not be observed after 4 h co-incubation, incubation with the HSP90α-inhibitor KUNA110 or the pan-HSP90 inhibitor 17AAG. Fig. 7C (right panels) shows similar results for TRAIL. Co-incubation with the HSP90β inhibitor also resulted in reduced HIF1α levels.

We also looked for other HSP90β downstream targets known to be involved in pro- and/or anti-apoptotic signaling. As shown in Fig. S1, further densitometric analysis of the apoptosis proteome profiler array revealed that after 8 h of TNF/Inhibitor stimulation, full length and cleaved caspase-3 and catalase appeared upregulated or stabilized, whereas in addition to HIF1α, FADD, HO-2 and HTRA2/Omi were reduced after inhibition of HSP90β. After 20 h, additionally claspin appeared reduced. Further densitometric analysis of the cell stress proteome profiler array revealed that in addition to HIF1α, Cited-2 is reduced after 8 h and Thioredoxin 1 after 20 h co-incubation (Fig. S2). These proteins have not been further investigated in this study.

## 3. Discussion

We and others previously showed that the signaling capacity of death receptors depends on their subcellular localization, which in turn appears to be regulated on their posttranslational modification status. Activated and M1-ubiquitinated TNF-R1 localized at the plasma membrane signals for cell survival via *complex I* formation. On the other side, K63-ubiquitinated TNF-R1 recruit the *complex II*/DISC proteins upon internalization of the receptor in TNF-receptosomes and thus, signal for cell death (Schütze et al., 2008; Brenner et al., 2015). Alternatively, shedding and RIPing of activated TNF-R1 may also activate cell death by DISC recruitment to cytosolic TNF-R1 (Chhibber-Goel et al., 2016). Further maturation of TNF-receptosomes towards a lysosomal compartment allows integration of the extrinsic and intrinsic death signaling pathways by permeabilization of both lysosomal and mitochondrial membranes resulting apoptosome formation and thus, amplification of the death signaling (for review, see Fritsch et al. (2017)). Similar signaling pathways have been described for TRAIL-R1 and TRAIL-R2 (for review, see Bertsch et al. (2014), van Roosmalen et al. (2014) and Lafont et al. (2018)).

One mediator of this lysosomal-mitochondrial amplification loop is CtsD, which is released from lysosomes to activate cytosolic proteins by proteolysis (i.e. Bid) or to deactivate anti-apoptotic proteins such as HSP90, as we recently described (Fritsch et al., 2016; Heinrich et al., 2004).

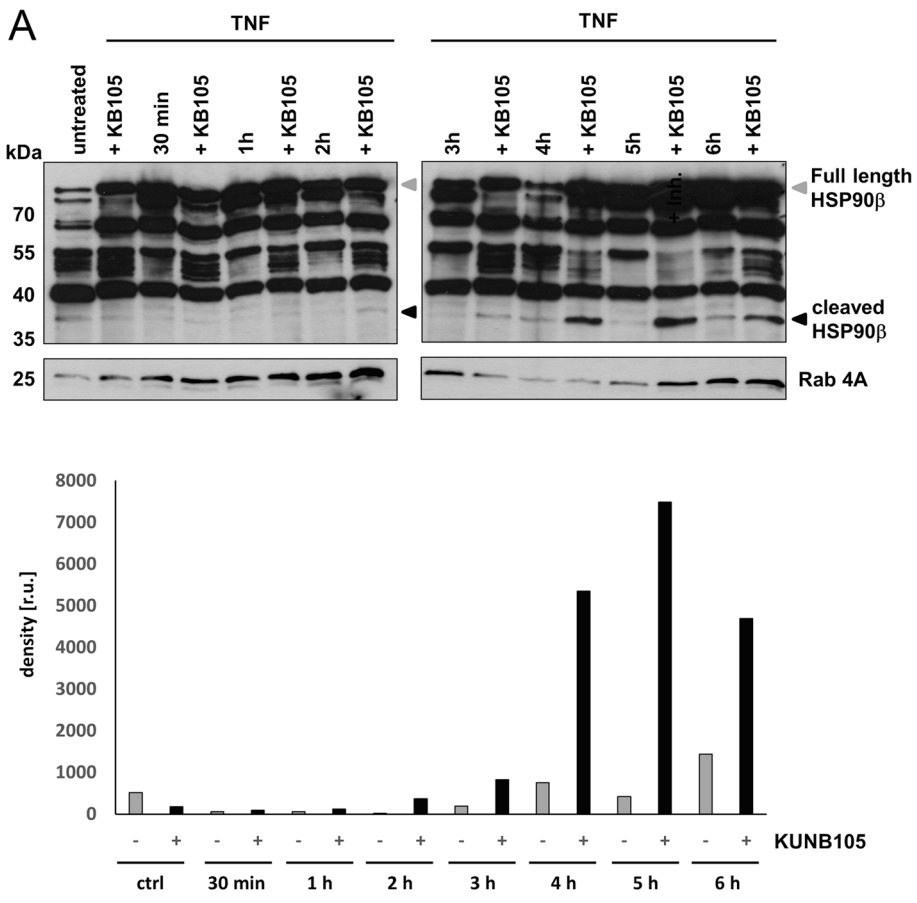
We here show, that activation of TNF-R1 or TRAIL-R1/-R2 by adding TNF or TRAIL, respectively, selectively results in HSP90β cleavage. Worth mentioning, besides appearance of the cleavage product we never observed decreasing levels of the full length protein. This suggests, that HSP90β is degraded only partially – putatively in distinct subcellular locations. Confocal laser scanning microscopy substantiated this assumption, revealing partial CtsD/HSP90 co-localization at the plasma membrane after 60 min of TNF stimulation. Such a change in HSP90 localization towards the plasma membrane has not been observed before and has to be analyzed in depth in future studies. One mechanism to trigger HSP90-plasma membrane localization of the otherwise cytosolic protein could be by palmitoylation. In our recent publication, we reported that signaling via TNF-R1 also required differential palmitoylation of various proteins (Zingler et al., 2019). In that study, HSP90 was one hit in a proteomics screen to identify proteins that are differentially palmitoylated in response to TNF. Such palmitoylation could be validated by acyl resin assisted capture of palmitoylated proteins and western blot, where HSP90 appears to be partially palmitoylated and 10 min incubation with TNF results in an altered band pattern compared to untreated cells as shown in Fig. S3. Detailed analyses have to be performed in future, to validate these preliminary observations. Similarly, it has to be investigated how CtsD is recruited to the PM. Again, palmitoylation of CtsD could be involved, as the protein has been identified twice in proteome analyses (Serwa et al., 2015; Hernandez et al., 2016). Functions of cathepsins at the PM have been reported before (Yadati et al., 2020).

Inhibition of HSP90β using the novel isoform specific inhibitor KUNB105, which targets the c-terminus of HSP90, boosted both TNF and TRAIL induced apoptosis. Intriguingly, usage of the HSP90 inhibitor Gambogic acid and its derivative DAP-19 did not reproduce this observation. This may be due to the different mode of action of the inhibitors targeting either the HSP90 middle domain (GBA and DAP-19) or the c-terminus (Yim et al., 2016). Thus, to exert its function in death receptor signaling, HSP90β obviously depends on its c-terminal region.

As co-incubation of TNF or TRAIL with KUNB105 resulted in increased apoptosis, comparable to the effect of CHX, we analyzed cleavage of PARP as well as caspase 8 and 9 in response to TNF or TRAIL combined with KUNB105. This revealed that in both caspases are activated. HSP90β is a known inhibitor of apaf-1 oligomerization/apoptosome formation (Bratton and Salvesen, 2010). This suggests, that both receptors enhance apoptosis induction via MOMP, which is in line with or previous report showing that HSP90 is cleaved by CtsD, resulting in enhanced caspase 9 activity and mitochondrial permeabilization upon Bid cleavage (Fritsch et al., 2016).

Both, TNF and TRAIL, activate translocation of NF-κB to the nucleus to allow transcription of anti-apoptotic proteins. We observed no alteration in IκB-degradation/NF-κB activation in response to either TNF or TRAIL.

As HSP90 stabilizes many anti-apoptotic proteins, we aimed to



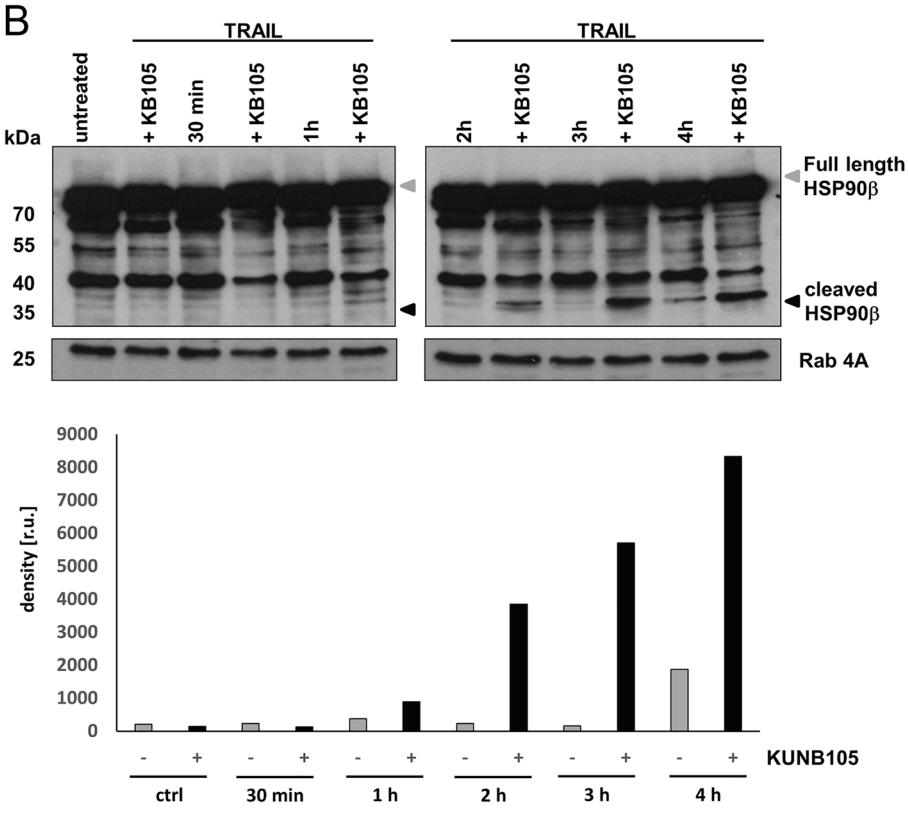




**Fig. 5.** Selective inhibition of HSP90β increases TNF and TRAIL induced HSP90β cleavage. U937 cells were treated for the indicated times with TNF or TRAIL and with the selective HSP90β inhibitor and compared to untreated lysate. A) Lysates after TNF ± Inhibitor treatment. WB was probed for HSP90β. B) Lysates after TRAIL ± Inhibitor treatment. WB was probed for HSP90β. Arrows indicate full length or cleaved HSP90. Rab4a serves as loading control. One representative of n = 3 experiments is shown. Densitometric analysis of the bands is shown below the respective WB (r.u = relative units).

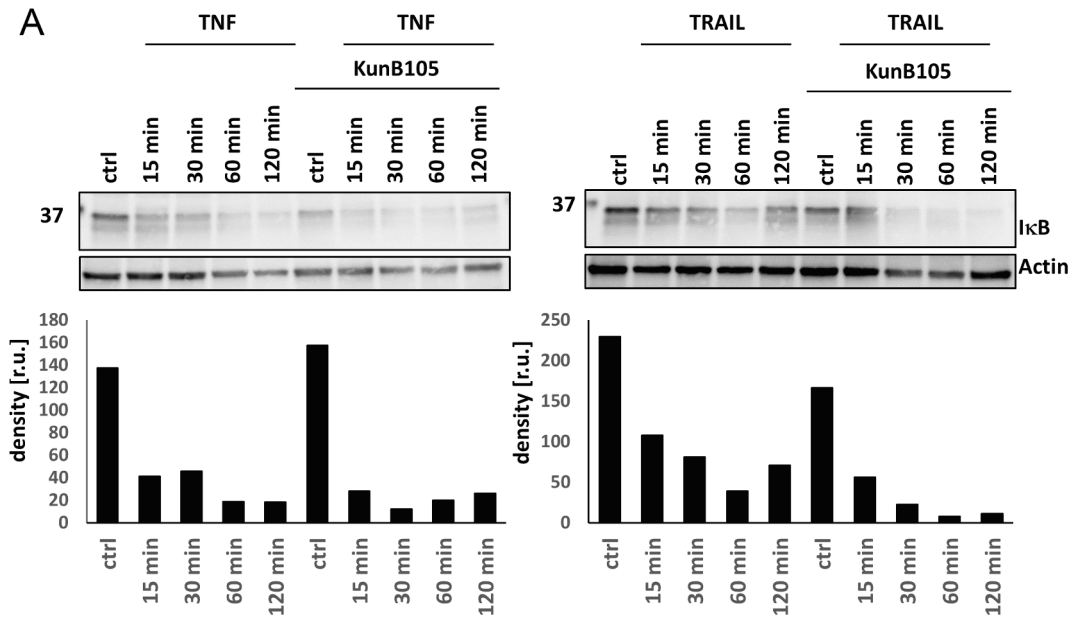



**Fig. 6.** Selective HSP90β inhibition does not affect TNF or TRAIL induced NF-κB. U937 cells were treated for the indicated times with TNF or TRAIL and KUNB105, as indicated. Cell lysates were probed for the presence if IκB. Actin served as loading control. One representative of n = 3 experiments is shown. Densitometric analysis of the bands is shown below the respective WB (r.u = relative units).

identify possible downstream targets which are affected by death ligand: HSP90β-inhibitor co-incubation. This way, we identified several proteins (i.e. HO-2, HTRA2/Omi, claspin, Cited-2, Thioredoxin 1 and HIF1α) that were reduced within 20 h co-incubation, while cleaved caspase 3 and catalase appeared upregulated or stabilized. Among these, the transcription factor HIF1α is the only target protein whose stability had been described to depend on HSP90 interaction and activity, before Kubo et al. (2004) and Kim et al. (2016). Classically, HIF1α is known to be upregulated/stabilized under hypoxia which frequently develops in solid tumor tissue due to rapid proliferation of tumor cells. By upregulating the transcription of various target genes, HIF1α mediates metabolism, cell death and survival, immune surveillance and genomic instability, resulting in tumor growth and metastasis (for review, see Xia et al. (2018) and Chen and Sang (2016)). Anti-TNF therapy is frequently applied to treat inflammatory diseases such as lupus or rheumatoid arthritis. Both HSP90 and HIF1α have also been suggested as therapeutic target for such diseases and thus, understanding the role of HIF1α in death receptor signaling may improve therapeutic options (Hua and Dias, 2016; Yang and Liu, 2016; Khandia et al., 2017). Cited-2 also appeared downregulated by TNF/HSP90β-inhibition. It is a transcriptional regulator induced by HIF1α and is involved to maintain homeostasis of several cellular key pathways (for review, see: Gezer et al. (2014)). Whether cited-2 is reduced due to HIF1α depletion or by another mode of action remains to be clarified.

Several reports showed that TRAIL mediated cell death induction is modulated by HIF1α and that resistance of cancer cells can be overcome by HIF1α inhibition (Knoll et al., 2016; Jeong et al., 2010; Lee et al., 2014). Discovery of TNF and FasL initially raised high hopes to target cancer, but revealed many tumor promoting activities (Sheng et al., 2018; Peter et al., 2015; Sheikh and Huang, 2004; French and Tschopp, 2003). Meanwhile, TRAIL is the most promising death ligand to target cancer, although also here, combination therapy with other drugs appears the best choice (Lim et al., 2015; Trivedi and Mishra, 2015; Yuan et al., 2018). Possible co-treatment with TRAIL and inhibition of mitochondrial HSP90 (TRAP-1/HSP90L) using Gamitrinib has been suggested by Siegelin et al. (2011).

Our presented findings are depicted in Fig. 8. In future, it has to be shown whether TNF/TRAIL:HSP90β inhibition results in i.e. HIF1α reduction due to decreased levels of the other proteins identified here, or vice versa, or if these effects occur independent form each other. Together, inhibition of HSP90β with simultaneous activation of death receptors appears to modulate also ROS production, resulting in enhanced apoptosis rates. The efficacy of such treatment has to be further evaluated in different cell and tissue models. In perspective, identification of HSP90β as major target for interfering with death receptor signaling is of special interest. Meanwhile, several clinical trials are running, testing pan-HSP90 small molecule inhibitors. These pan-inhibitors often show toxicity in various organs, partially by inducing massive heat shock response via other HSP molecules (Khandelwal et al., 2016, 2018). Thus, selective targeting of only one HSP90 isoform in combination with death receptor ligation may provide alternative routes for the treatment of tumors and other diseases.

## 4. Materials and methods

### 4.1. Reagents and antibodies

Isoform selective HSP90 inhibitors (KUNB105, KUNA110; both unpublished) were provided by BSJ Blagg. *Killer*TRAIL™ (ALX-201-073-3020) was purchased from Enzo, TNF was a gift by D. Männel (Regensburg).

Primary antibodies used in this study: HSP90 antibodies were from were from ThermoFisher Scientific: anti-HSP90α (PA3-013) anti-HSP90β (PA3-012). HRP-conjugated anti-tubulin antibody (HRP-66031) and anti-GAPDH (HRP-60004) were from Proteintech. Anti-Rab4A (sc-312) was from Santa Cruz Biotechnology. Anti-IκB (#4814), anti-clPARP (#9541), anti-Casp9 (#7237) and anti-HIF1α (#14179S) was from Cell Signaling. anti-Caspase 8 (ALX-804-242) was from Enzo.

For fluorescence microscopy: anti-HSP90 (#4874, Cell Signaling), anti-Cathepsin D (ab6313, Abcam).

Secondary antibodies used in this study: anti-mouse light chain HRP conjugated (AP200P) and anti-rabbit light chain HRP conjugated (MAB201P) from Millipore. For fluorescence microscopy: anti-mouse Alexafluor 488, anti-rabbit Alexafluor 555 (A21202 and A31572, Invitrogen)

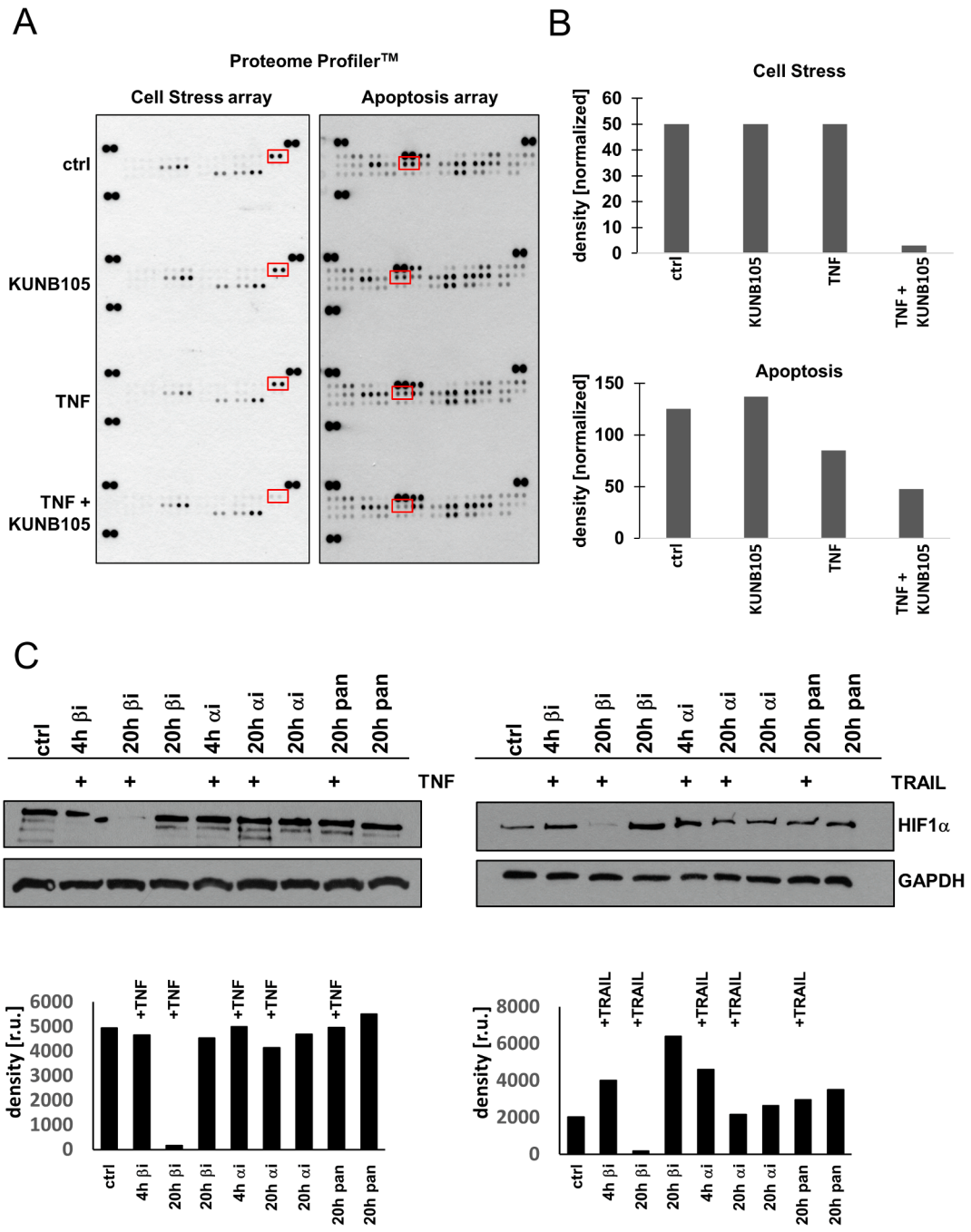



**Fig. 7.** Selective inhibition of HSP90β results in TNF and TRAIL mediated HIF1α degradation. A) Proteome profiler™ analysis: U937 cells were treated for 20 h with TNF or TRAIL and with KUNB105 and compared to untreated lysate (ctrl). Left panel: Cell Stress array; Right panel: Apoptosis array. Changes in HIF1α abundance in both arrays is indicated by the red box. B) Quantification of HIF1α spot intensity by densitometry. Upper panel: Cell Stress array; Lower panel: Apoptosis array. Analysis of HIF1α degradation upon stimulation with: C) TNF (left panels), TRAIL (right panels) in total cell lysates in the presence of KUNB105, KUNA110 or 17AAG. GAPDH serves as loading control. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

### 4.2. Cell culture

U937 and HeLa were obtained from DSMZ (Braunschweig) and cultured in RPMI 1640 and DMEM respectively, supplemented with 5% FCS (Biochrom) and penicillin/streptomycin (Biochrom).

### 4.3. SDS-PAGE and western blotting

Cell lysates were prepared using a modified RIPA buffer (50 mM TRIS-HCl [pH 7.5], 150 mM NaCl, 1% NP-40, 1% Triton X-100, 1 mM EDTA, 0.25% Na-deoxycholate), containing protease inhibitor cocktail (Roche) and protein concentration was determined by BCA (Pierce). For SDS-PAGE 12.5% PAA gels were used. Proteins were blotted to PVDF membrane (Carl-Roth). The membranes were blocked with 5% skimmed milk in TBST and incubated over night with the primary antibody diluted 1:500–1:5000 in 5% skimmed milk. The peroxidase conjugated secondary antibodies were incubated for 1 h diluted 1:10000 in 5% skimmed milk. Blots were developed using the ECL kit (RPN2106) and films or the LAS4000mini and ECL kit (RPN2236) from GE Healthcare. Bands were scanned using an Epson Perfection V330 scanner and quantified using ImageJ.

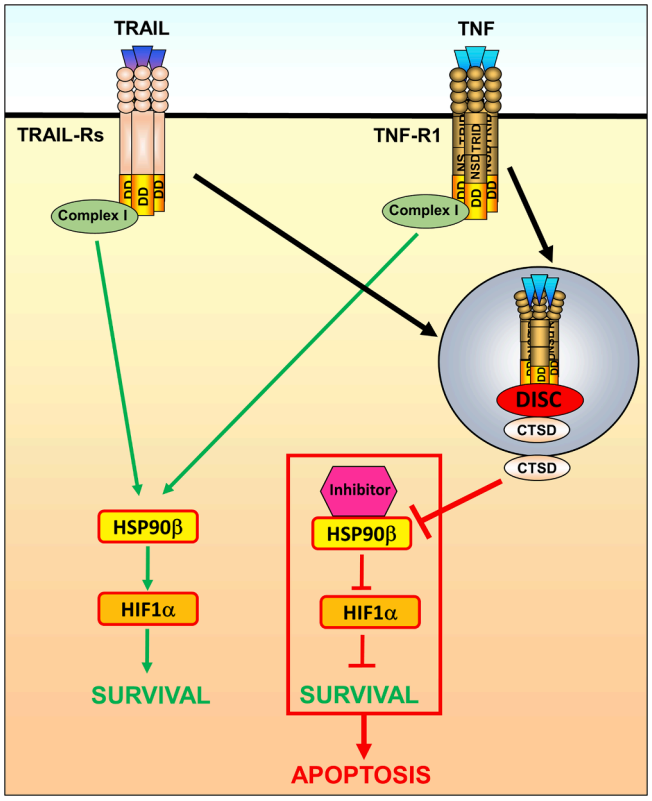



**Fig. 8.** Model. Surface resident TNF-R1 or TRAIL-Rs activate NF-κB signaling via *Complex I* formation. Internalized TNF-R1 or TRAIL-Rs trigger HSP90β degradation and HIF1α destabilization in combination with inhibitor (KUNB105) treatment.

### 4.4. Image stream apoptosis assay

For apoptosis measurement, cells were incubated for the times indicated in the figure with the respective death ligand (100 ng/ml) under standard cell culture conditions. 30 min before end Hoechst stain (Sigma-Aldrich) was added to the culture medium, finally diluted 1:10000. Up to 10000 images were acquired, detecting the nuclear stain (excitation: 405 nm) at Channel 1. For the image acquisition, the 60x objective was used. The apoptosis wizard was used for assaying the number of cells showing nuclear fragmentation compared to cells with intact nuclei.

### 4.5. Guava MUSE cell death assay

For the assay, $1 \times 10^6$ cells were incubated with ligand and inhibitors in 1 ml growth medium over night at standard cell culture conditions. For staining, 25 µl of the cell suspension were mixed with 25 µl of the Annexin V/7-AAD reagent provided in the kit (MCH100105), and incubated for 20 min in the dark, before adding 150 µl PBS. Measurement was performed using the Guava MUSE, according to the manufacturer's instructions.

### 4.6. Proteome profiler

The Proteome Profiler arrays (Human Cell Stress Array Kit: #ARY018; Human Apoptosis Array Kit: #ARY009, bio-techne) were performed as recommended by the manufacturer. For quantification, the membranes were scanned using an Epson Perfection V330 scanner and quantified using ImageJ software.

### 4.7. Fluorescence microscopy

Cells were grown on fibronectin-coated coverslips (#354088, Corning) and incubated for 60 min at 4 °C with TNF (100 ng/ml). The temperature was shifted to 37 °C with pre-warmed medium for the times indicated in the figure legend to allow receptor internalization. Subsequently, cells were fixed in 4% paraformaldehyde (PFA)/PBS for 20 min, permeabilized in PBS supplemented with 0.1% saponin and 0.2% bovine serum albumin (BSA), and stained with the respective primary antibodies at a 1:50 dilution for 60 min at room temperature, followed by incubation with the respective secondary antibodies. Immunofluorescence analysis was performed with a confocal laser scanning microscope (LSM 510, equipped with an Axiovert 100 M; Zeiss). All LSM micrographs were acquired with a 63× objective.

### 4.8. Acyl resin assisted capture (AcylRAC)

AcylRAC was performed as recently described (Fritsch et al., 2016): $1 \times 10^8$ cells per sample were incubated with 100 ng/ml of TNF for 15 min on ice, followed by warming up to 37 °C for the indicated times. Cold PBS was added and cells were sedimented, followed by lysis in 1 ml buffer A (25 mM HEPES [pH 7.4], 25 mM NaCl, 1 mM EDTA, PIC) using sonication (45 s, constant output 2.5, 4 °C) (G. Heinemann, Germany). An aliquot was stored as input in lysis buffer (50 mM TRIS-HCl [pH 7.5], 150 mM NaCl, 1% NP-40, 1% Triton X-100, 1 mM EDTA, 0.25% Na-deoxycholate). Debris was removed by 2× centrifugation (800×*g*, 5 min, 4 °C) followed by membrane sedimentation for 50 min at 4 °C at 16,200×*g*. The resulting pellet was resuspended in buffer A/0.5% Triton X-100. 1.5 mg protein solution was mixed with the blocking solution (100 mM HEPES [pH 7.5], 1 mM EDTA, 2.5% SDS, 2.5% MMTS (Sigma-Aldrich)) in a 1:2 ratio at 40 °C for 2 h, followed by acetone precipitation. The precipitate was resuspended in 400 µl binding buffer (100 mM (Schütze et al., 2008) HEPES [pH 7.5], 1 mM EDTA, 1% SDS), split equally and added to 0.05 g activated thiopropyl sepharose 6B (GE Healthcare) in binding buffer. One part was treated with hydroxylamine [pH 7.5] the other part with Tris-HCl [pH 7.5], final concentration 0.5 M each. After overnight incubation, beads were washed and used for SDS-PAGE.

## 5. Author's contributions

ALH performed most experiments as part of her MD thesis. TP, LF, EA, VS performed experiments. SM and BSJB provided HSP90 inhibitors. WSB provided reagents. SS supervised the project. JF planned and supervised the project and wrote the manuscript. All authors read and revised the manuscript.

## Funding

ALH was supported by the *Integrated Research Training Group* of the DFG CRC877. SS received funding by the DFG (CRC877 project B1 and Schu733/14-1).

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.imbio.2021.152070.

## References


Schütze, S., Tchikov, V., Schneider-Brachert, W., 2008. Regulation of TNFR1 and CD95 signalling by receptor compartmentalization. Nat. Rev. Mol. Cell Biol. 9 (8), 655–662.

Mahul-Mellier, A.L., et al., 2008. Alix and ALG-2 are involved in tumor necrosis factor receptor 1-induced cell death. J. Biol. Chem. 283 (50), 34954–34965.

Chhibber-Goel, J., et al., 2016. gamma-secretase activity is required for regulated intramembrane proteolysis of tumor necrosis factor (TNF) receptor 1 and TNF-mediated pro-apoptotic signaling. J. Biol. Chem. 291 (11), 5971–5985.

Holdbrooks, A.T., Britain, C.M., Bellis, S.L., 2018. ST6Gal-I sialyltransferase promotes tumor necrosis factor (TNF)-mediated cancer cell survival via sialylation of the TNF receptor 1 (TNFR1) death receptor. J. Biol. Chem. 293 (5), 1610–1622.

Ali, M., et al., 2013. Altering the sphingolipid acyl chain composition prevents LPS/GLN-mediated hepatic failure in mice by disrupting TNFR1 internalization. Cell Death Dis. 4.

Fritsch, J., et al., 2014. Cell fate decisions regulated by K63 ubiquitination of tumor necrosis factor receptor 1. Mol. Cell. Biol. 34 (17), 3214–3228.

Schneider-Brachert, W., et al., 2006. Inhibition of TNF receptor 1 internalization by adenovirus 14.7K as a novel immune escape mechanism. J. Clin. Invest. 116 (11), 2901–2913.

Schneider-Brachert, W., et al., 2004. Compartmentalization of TNF receptor 1 signaling: internalized TNF receptosomes as death signaling vesicles. Immunity 21 (3), 415–428.

Zhang, Y., Yoshida, T., Zhang, B., 2009. TRAIL induces endocytosis of its death receptors in MDA-MB-231 breast cancer cells. Cancer Biol. Ther. 8 (10), 917–922.

Akazawa, Y., et al., 2009. Death receptor 5 internalization is required for lysosomal permeabilization by TRAIL in malignant liver cell lines. Gastroenterology 136 (7), pp. 2365–2376 e1–7.

Mazurek, N., et al., 2012. Cell-surface galectin-3 confers resistance to TRAIL by impeding trafficking of death receptors in metastatic colon adenocarcinoma cells. Cell Death Differ. 19 (3), 523–533.

Austin, C.D., et al., 2006. Death-receptor activation halts clathrin-dependent endocytosis. Proc. Natl. Acad. Sci. U.S.A. 103 (27), 10283–10288.

Kohlhaas, S.L., et al., 2007. Receptor-mediated endocytosis is not required for tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis. J. Biol. Chem. 282 (17), 12831–12841.

Sosna, J., et al., 2016. Differences and similarities in TRAIL- and tumor necrosis factor-mediated necroptotic signaling in cancer cells. Mol. Cell. Biol. 36 (20), 2626–2644.

Fritsch, J., et al., 2016. TNF induced cleavage of HSP90 by cathepsin D potentiates apoptotic cell death. Oncotarget 7 (46), 75774–75789.

Kampinga, H.H., et al., 2009. Guidelines for the nomenclature of the human heat shock proteins. Cell Stress Chaperones 14 (1), 105–111.

Verma, S., et al., 2016. Hsp90: friends, clients and natural foes. Biochimie 127, 227–240.

Calderwood, S.K., Gong, J., 2016. heat shock proteins promote cancer: it's a protection racket. Trends Biochem. Sci. 41 (4), 311–323.

Pandey, M.K., et al., 2016. Targeting cell survival proteins for cancer cell death. Pharmaceuticals (Basel) 9 (1).

Garg, G., Khandelwal, A., Blagg, B.S., 2016. Anticancer inhibitors of Hsp90 function: beyond the usual suspects. Adv. Cancer Res. 129, 51–88.

Khandelwal, A., Crowley, V.M., Blagg, B.S.J., 2016. Natural product inspired N-terminal Hsp90 inhibitors: from bench to bedside? Med. Res. Rev. 36 (1), 92–118.

Prasad, S., et al., 2014. Targeting death receptors for TRAIL by agents designed by mother nature. Trends Pharmacol. Sci. 35 (10), 520–536.

Lim, B., et al., 2015. Targeting TRAIL in the treatment of cancer: new developments. Expert Opin. Ther. Targets 19 (9), 1171–1185.

Trivedi, R., Mishra, D.P., 2015. Trailing TRAIL resistance: novel targets for TRAIL sensitization in cancer cells. Front. Oncol. 5, 69.

Sanchez, J., et al., 2020. Old and new approaches to target the Hsp90 chaperone. Curr. Cancer Drug Targets 20 (4), 253–270.

Edelmann, B., et al., 2011. Caspase-8 and caspase-7 sequentially mediate proteolytic activation of acid sphingomyelinase in TNF-R1 receptosomes. EMBO J. 30 (2), 379–394.

Heinrich, M., et al., 2004. Cathepsin D links TNF-induced acid sphingomyelinase to Bid-mediated caspase-9 and -3 activation. Cell Death Differ. 11 (5), 550–563.

Liu, Y.V., Semenza, G.L., 2007. RACK1 vs. HSP90: competition for HIF-1 alpha degradation vs. stabilization. Cell Cycle 6 (6), 656–659.

Brenner, D., Blaser, H., Mak, T.W., 2015. Regulation of tumour necrosis factor signalling: live or let die. Nat. Rev. Immunol. 15 (6), 362–374.

Fritsch, J., et al., 2017. Role of ubiquitination and proteolysis in the regulation of pro- and anti-apoptotic TNF-R1 signaling. Biochim. Biophys. Acta Mol. Cell Res. 1864 (11 Part B), 2138–2146.

Bertsch, U., et al., 2014. Compartmentalization of TNF-related apoptosis-inducing ligand (TRAIL) death receptor functions: emerging role of nuclear TRAIL-R2. Cell Death Dis. 5, e1390.

van Roosmalen, I.A., Quax, W.J., Kruyt, F.A., 2014. Two death-inducing human TRAIL receptors to target in cancer: similar or distinct regulation and function? Biochem. Pharmacol. 91 (4), 447–456.

Lafont, E., Hartwig, T., Walczak, H., 2018. Paving TRAIL's path with ubiquitin. Trends Biochem. Sci. 43 (1), 44–60.

Zingler, P., et al., 2019. Palmitoylation is required for TNF-R1 signaling. Cell Commun. Signal. 17 (1), 90.

Serwa, R.A., et al., 2015. Systems analysis of protein fatty acylation in herpes simplex virus-infected cells using chemical proteomics. Chem. Biol. 22 (8), 1008–1017.

Hernandez, J.L., et al., 2016. Correlated S-palmitoylation profiling of Snail-induced epithelial to mesenchymal transition. Mol. BioSyst. 12 (6), 1799–1808.

Yadati, T., et al., 2020. The Ins and outs of cathepsins: physiological function and role in disease management. Cells 9 (7).

Yim, K.H., et al., 2016. Gambogic acid identifies an isoform-specific druggable pocket in the middle domain of Hsp90beta. Proc. Natl. Acad. Sci. U.S.A. 113 (33), E4801–E4809.

Bratton, S.B., Salvesen, G.S., 2010. Regulation of the Apaf-1-caspase-9 apoptosome. J. Cell Sci. 123 (Pt 19), 3209–3214.

Kubo, T., et al., 2004. Bisphenol A, an environmental endocrine-disrupting chemical, inhibits hypoxic response via degradation of hypoxia-inducible factor 1alpha (HIF-1alpha): structural requirement of bisphenol A for degradation of HIF-1alpha. Biochem. Biophys. Res. Commun. 318 (4), 1006–1011.

Kim, H.S., et al., 2016. Synthesis and biological evaluation of C-ring truncated deguelin derivatives as heat shock protein 90 (HSP90) inhibitors. Bioorg. Med. Chem. 24 (22), 6082–6093.

Xia, Y., Jiang, L., Zhong, T., 2018. The role of HIF-1alpha in chemo-/radioresistant tumors. Onco Targets Ther. 11, 3003–3011.

Chen, S., Sang, N., 2016. Hypoxia-inducible factor-1: a critical player in the survival strategy of stressed cells. J. Cell. Biochem. 117 (2), 267–278.

Hua, S., Dias, T.H., 2016. Hypoxia-inducible factor (HIF) as a target for novel therapies in rheumatoid arthritis. Front. Pharmacol. 7, 184.

Yang, Z.-C., Liu, Y., 2016. Hypoxia-inducible factor-1α and autoimmune lupus, arthritis. Inflammation 39 (3), 1268–1273.

Khandia, R., et al., 2017. Heat shock proteins: therapeutic perspectives in inflammatory disorders. Recent Pat. Inflamm. Allerg. Drug Discov. 10 (2), 94–104.

Gezer, D., et al., 2014. Concise review: genetic dissection of hypoxia signaling pathways in normal and leukemic stem cells. Stem Cells 32 (6), 1390–1397.

Knoll, G., et al., 2016. Hypoxia regulates TRAIL sensitivity of colorectal cancer cells through mitochondrial autophagy. Oncotarget 7 (27), 41488–41504.

Jeong, J.K., et al., 2010. Hypoxia inducing factor-1alpha regulates tumor necrosis factor-related apoptosis-inducing ligand sensitivity in tumor cells exposed to hypoxia. Biochem. Biophys. Res. Commun. 399 (3), 379–383.

Lee, Y.J., et al., 2014. Overcoming hypoxic-resistance of tumor cells to TRAIL-induced apoptosis through melatonin. Int. J. Mol. Sci. 15 (7), 11941–11956.

Sheng, Y., Li, F., Qin, Z., 2018. TNF receptor 2 makes tumor necrosis factor a friend of tumors. Front. Immunol. 9, 1170.

Peter, M.E., et al., 2015. The role of CD95 and CD95 ligand in cancer. Cell Death Differ.

Sheikh, M.S., Huang, Y., 2004. Death receptors as targets of cancer therapeutics. Curr. Cancer Drug Targets 4 (1), 97–104.

French, L.E., Tschopp, J., 2003. Protein-based therapeutic approaches targeting death receptors. Cell Death Differ. 10 (1), 117–123.

Yuan, X., et al., 2018. Developing TRAIL/TRAIL death receptor-based cancer therapies. Cancer Metastasis Rev.

Siegelin, M.D., et al., 2011. Exploiting the mitochondrial unfolded protein response for cancer therapy in mice and human cells. J. Clin. Invest. 121 (4), 1349–1360.

Khandelwal, A., et al., 2018. Structure-guided design of an Hsp90beta N-terminal isoform-selective inhibitor. Nat. Commun. 9 (1), 425.