

Regulation of PIDD auto-proteolysis and activity by the molecular chaperone Hsp90

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In response to DNA damage, p53-induced protein with a death domain (PIDD) forms a complex called the PIDDosome, which either consists of PIDD, RIP-associated protein with a death domain and caspase-2, forming a platform for the activation of caspase-2, or contains PIDD, RIP1 and NEMO, important for NF- κ B activation. PIDDosome activation is dependent on auto-processing of PIDD at two different sites, generating the fragments PIDD-C and PIDD-CC. Despite constitutive cleavage, endogenous PIDD remains inactive. In this study, we screened for novel PIDD regulators and identified heat shock protein 90 (Hsp90) as a major effector in both PIDD protein maturation and activation. Hsp90, together with p23, binds PIDD and inhibition of Hsp90 activity with geldanamycin efficiently disrupts this association and impairs PIDD auto-processing. Consequently, both PIDD-mediated NF- κ B and caspase-2 activation are abrogated. Interestingly, PIDDosome formation itself is associated with Hsp90 release. Characterisation of cytoplasmic and nuclear pools of PIDD showed that active PIDD accumulates in the nucleus and that only cytoplasmic PIDD is bound to Hsp90. Finally, heat shock induces Hsp90 release from PIDD and PIDD nuclear translocation. Thus, Hsp90 has a major role in controlling PIDD functional activity.

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Initiator caspases are commonly activated in high molecular weight complexes containing a protein with an activation-induced oligomerisation capacity that will in turn recruit the initiator caspase and induce its activation.¹ The activation platform for caspase-2 has long remained enigmatic until the PIDDosome was identified, a complex consisting of PIDD (p53-induced protein with a death domain; LRDD), the adaptor protein RAIDD (RIP-associated protein with a death domain; CRADD) and caspase-2.^{2–4} PIDD can also recruit RIP1 and NEMO in response to DNA damage, leading to NF- κ B activation^{5,6} and was also recently described in a nuclear complex with DNA-PKcs and caspase-2, formed in response to γ -irradiation having a role in DNA repair and cell cycle checkpoint maintenance.⁷

The opposing signalling functions of PIDD, namely its pro-survival (NF- κ B activation) and pro-death (caspase-2 activation) functions, were shown to be dependent on different active fragments of PIDD, generated by auto-processing.⁶ Although a first processing event at S446 generates PIDD-N (1–445, containing the N-terminus and the leucine-rich repeats, LRR) and PIDD-C (446–910, containing the C-terminus and the death-domain, DD), the second cleavage event at S588 produces the proapoptotic fragment PIDD-CC (588–910, containing the DD). PIDD-C binds RIP1 and NEMO controlling the pro-survival NF- κ B arm, whereas PIDD-CC

together with RAIDD activates the apoptotic caspase-2. PIDD-N has been suggested to act as a regulatory fragment.

In unstimulated cells, both active fragments of PIDD can be readily detected because of constitutive auto-processing of the full-length protein,^{6,8,9} but no activation of NF- κ B or caspase-2 can be observed. In order to understand the mechanism of how PIDD activity is regulated, we screened for potential novel PIDD regulators and found many representatives of the molecular chaperone machinery. In this study, we focused on heat shock protein 90 (Hsp90) as a potential regulatory interactor of PIDD. Molecular chaperones are characterised by their essential roles in protein folding, stability and maturation. In addition, several studies have reported the involvement of Hsp90, in other cellular processes such as apoptosis.¹⁰ Moreover, NOD1, a protein that shares common domain organisation with the core protein of initiator caspase activating platforms, was recently shown to be regulated by Hsp90.¹¹

Results

PIDD interacts with the molecular chaperone machinery. In recent years PIDD was found to be part of different multi-molecular regulatory complexes named PIDDosomes.^{4,5,7} In search of PIDD interaction partners,

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Abbreviations: PIDD, p53-induced protein with a death domain; RAIDD, RIP-associated protein with a death domain; HSP, heat shock protein; GA, geldanamycin; CHIP, carboxy terminus of HSP70-interacting protein; CHX, cyclohexamide

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we performed a yeast-two hybrid and a proteomics screen. Both screens yielded high hit rates suggesting that PIDD is a nodal and highly connected protein. We found that PIDD appeared to be associate readily with many representatives of the molecular chaperone machinery including both the α and β isoforms of Hsp90 (Table 1). The interactions of PIDD with Hsp90, its co-chaperone p23 and Hsp70 were confirmed in immunoprecipitates of HEK293T lysates stably expressing PIDD, also at the endogenous level (Figure 1a and b).

Table 1 Molecular chaperones found in PIDD interaction screens

Hsp90	Heat shock protein 90 ($\alpha+\beta$)
Hsp70	Heat shock protein 70
p23	Co-chaperone of Hsp90
CHIP	STUB1; Carboxy terminus of Hsp70-interacting protein
Hop	STIP1; Hsc70/Hsp90-organizing protein
DNAJ	Several members of the A, B and C subfamilies were detected
BAG	Members -2 and -5 were detected

Abbreviaton: PIDD, p53-induced protein with a death domain

To test the contribution of the co-chaperone p23 (critical for Hsp90-client stabilisation¹²) in this interaction, p23 was downregulated using siRNA and PIDD was immunoprecipitated. We observed a reduction in Hsp90-PIDD complex formation, but not with Hsp70, suggesting that p23 has a stabilising role for the Hsp90-PIDD interaction only (Figure 1c). We further investigated these complexes by using geldanamycin (GA), a well studied inhibitor of Hsp90 activity.¹³ The results show an efficient and rapid disruption of the Hsp90/p23 complex, indicating that p23 depends on Hsp90 for complex inclusion. In contrast, Hsp70 remained associated with PIDD following GA treatment (Figure 1d). The co-chaperone CHIP (carboxy terminus of Hsp70-interacting protein; STUB1), which also associates with PIDD (Supplementary Figure 1A), was like Hsp70 not affected by the treatment (Supplementary Figure 1B). CHIP contains a U-box domain, which is similar to the RING-finger motifs found in ubiquitin ligases.¹⁴ The ubiquitin ligase functions of CHIP are well described and it is thought that it uses chaperones such as Hsp70 as substrate-recognition components.¹⁵

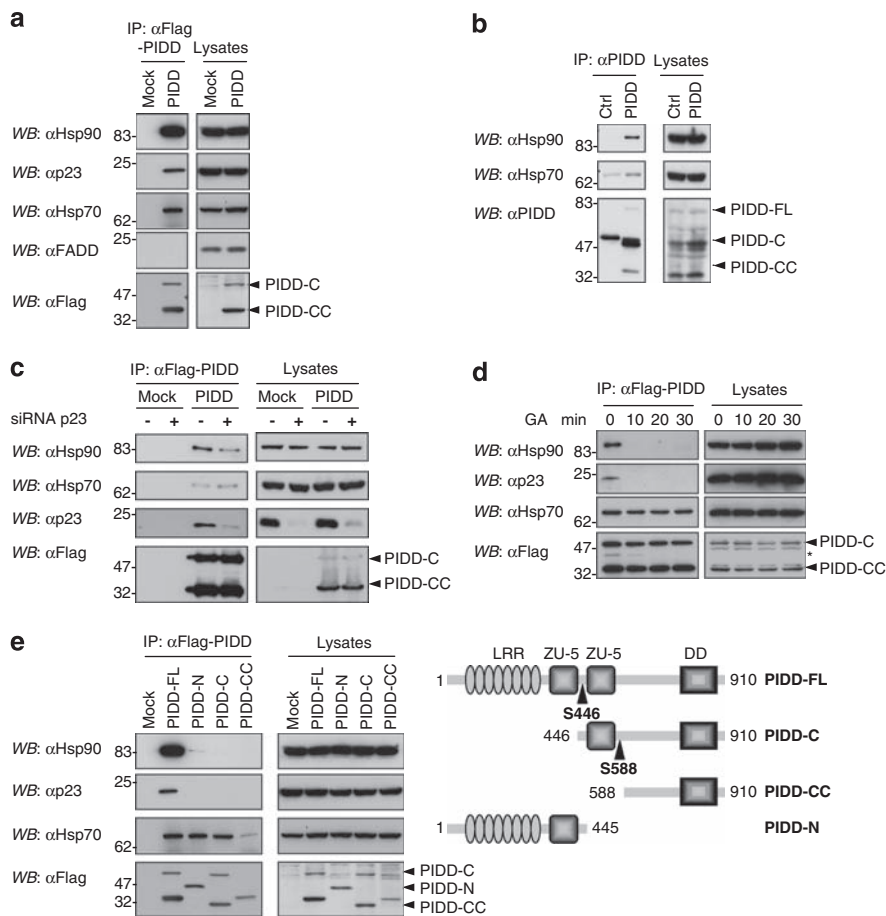


Figure 1 Hsp90, p23 and Hsp70 interact with PIDD. (a) Anti-Flag immunoprecipitation was performed with HEK293T cells stably expressing Flag-tagged PIDD or infected with empty vector (mock) and analysed by western blotting. (b) Endogenous immunoprecipitation using anti-PIDD or a control antibody (ctrl) on HEK293T lysates and analysed by western blotting. (c) Immunoprecipitation of Flag-tagged PIDD stably expressed in HEK293T cells or empty vector-containing control cells (mock) transfected with p23-specific siRNA (+) or a scrambled control (-). Analysis was performed by western blotting. (d) HEK293T cells stably expressing PIDD were treated with 1 μ M GA for the indicated times followed by an anti-Flag immunoprecipitation and analysed by western blotting. (e) Same as (a) using different Flag-tagged PIDD fragments. Slight differences in size observed with PIDD-CC are due to the cloning strategy of the tag-linker region employed. Schematic representation of the PIDD constructs used. Molecular weight masses are indicated in kDa

In order to determine the site of interaction between PIDD and Hsp90 in more detail, we expressed fragments of PIDD corresponding to the auto-cleavage products. Interestingly, the presence of both PIDD-N and PIDD-C is required for Hsp90 binding as neither fragment alone was found to interact with Hsp90 (Figure 1e, Supplementary Figure 1C and D). The same pattern of interaction was observed for p23. However, Hsp70 was able to associate with PIDD, as well as PIDD-N and PIDD-C. We further tested the interaction of the three different isoforms that have been described for PIDD with Hsp90, p23 and Hsp70. The results show a strong interaction of Hsp90 with isoform 1 only, whereas Hsp70 interacted strongly with isoform 2, as well as, but less so, with isoforms 1 and 3 (Supplementary Figure 1E). This may suggest that the interactions of Hsp70 and Hsp90/p23 with PIDD occur independently and possibly reflect different functional binding activities.

PIDD stability is regulated by Hsp90 and CHIP. In a fashion similar to other Hsp90 client proteins like IRF-1 (interferon regulators factor-1),¹⁶ both GA and Radicol (an alternative Hsp90 inhibitor¹⁷) treatments led to a dramatic decrease of PIDD protein levels (Figure 2a and Supplementary Figure 2A, respectively). Noteworthy, the half-life of PIDD was around 2 h during GA treatment, whereas other Hsp90 client such as IKK α and RIP1^{18,19} were not markedly affected after 8 h of treatment (Figure 2a and Supplementary Figure 2B). In addition, PIDD-CC appeared more stable in response to GA. The sensitivity of PIDD-C to GA was also observed in other cell lines (Supplementary Figure 2B and data not shown) and suggests a high dependence of PIDD on Hsp90 for its stability. PIDD levels were not affected by cycloheximide (CHX) treatment during a similar time period, demonstrating that the effect seen is not due to a constitutively short half-life, in comparison to cFLIP, which is rapidly turned over (Figure 2b).

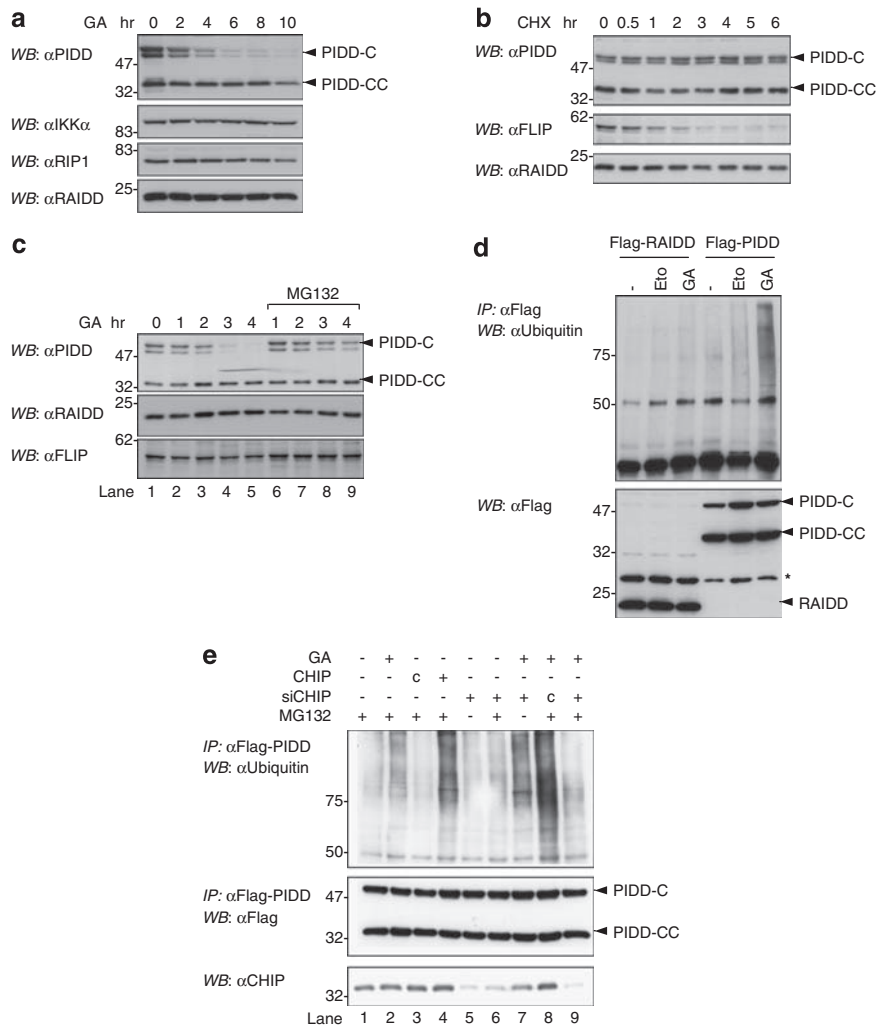


Figure 2 Geldanamycin induces rapid proteasome-dependent degradation of PIDD. (a) Raji B cells were treated with 1 μ M GA for the indicated times and analysed by western blotting. (b) Same as (a), but using cycloheximide (CHX) 10 μ g/ml treatment. (c) Same as (a) with 20 min of MG132 (10 μ M) pretreatment, where indicated. (d) Immunoprecipitation of Flag-tagged RAIDD or PIDD stably expressed in HEK293T cells treated with etoposide (3 h, 40 μ M), GA (1 h, 1 μ M) or DMSO control (-) in the presence of MG132 (10 μ M) and analysed by western blotting. (e) Immunoprecipitation of Flag-tagged PIDD stably expressed in HEK293T cells treated, where indicated, with GA (1 h, 1 μ M), MG132 (2 h, 10 μ M) and transfected with CHIP, CHIP-specific siRNA or the corresponding controls (c) and analysed by western blotting. Molecular weight masses are indicated in kDa and non-specific bands are marked by the asterisk

In agreement with previous studies on the stability of Hsp90 clients,^{16,18} PIDD degradation in response to GA also occurs in a mainly proteasome-dependent manner, as shown by the inhibitory effect of MG132 (Figure 2c, lane 4,5 versus 8,9). Although over time PIDD degradation is also detected in the presence of MG132 (Figure 2c, lane 6–9). Previously demonstrated with *c-erbB-2*, for example,²⁰ Hsp90 substrates are often ubiquitinated in response to GA preceding their degradation, which, as shown in Figure 2d, is also the case for PIDD. Considering the interaction between PIDD and CHIP (Supplementary Figure 1B), we further investigated the involvement of the ubiquitin ligase and co-chaperone CHIP in the ubiquitination of PIDD in response to GA. It has been shown previously that CHIP can ubiquitinate client proteins such as LRKK2 (LRR kinase 2) leading to its degradation.²¹ Overexpression of CHIP alone induced PIDD ubiquitination (Figure 2e; lane 3 versus 4), whereas downregulation of CHIP using siRNA reduced the GA-induced modification (Figure 2e; lane 8 versus 9).

PIDD auto-processing requires an Hsp90-supported conformation. Heat shock proteins have long been known to have an important role in protein folding, supporting a conformation important for activity, as for example in the case of steroid receptors.²² Using an inducible expression system (Flp-In T-Rex) for PIDD in HEK293T (HEK293T-Rex), we could observe a critical function for Hsp90 in the auto-processing function of PIDD. Figure 3a shows the accumulation of full-length PIDD upon induction when the cells were treated with GA. As previously reported, inhibition of protein synthesis by CHX treatment led to the conversion of PIDD to PIDD-C and finally to the accumulation of PIDD-CC in HEK293T cells stably expressing PIDD (Figure 3b and 6). Simultaneous treatment with GA, however, retarded the disappearance of PIDD-C and the

subsequent accumulation of PIDD-CC (Figure 3b lane 3 versus 9). We confirmed this observation in an *in vitro* auto-processing system using the PIDD F445H mutant,^{6,23} where induction of processing generating the PIDD-C (PIDD-CC not affected) by the addition of hydroxylamine was abrogated in the presence of GA (Figure 3c).

Thus, GA inhibits cleavage of PIDD at both S446 and S588 sites, suggesting that the conformation of PIDD is altered in the absence of Hsp90 and does no longer support auto-processing. These findings suggest that optimal PIDD conformation, mediated by the chaperone function of Hsp90, is essential for auto-cleavage.

Hsp90 is released upon PIDD activation. Hsp90 has been shown to act as a regulator of nuclear receptors as well as of several proteins involved in the apoptotic and inflammatory process. A general feature of this interaction is the release of Hsp90 from its client upon activation, which has been previously demonstrated, for example, in the case of PKR.²² To investigate whether the interaction of PIDD with heat shock proteins is affected under activating conditions, we took advantage of a cell-free system previously established.^{4,5} Hypotonic lysates from HEK293T cells stably expressing Flag-tagged PIDD were exposed to 37°C, which induces PIDosome complex formation. The immunoprecipitation (IP) showed a rapid release of Hsp90 and p23 from PIDD (Figure 4a and Supplementary Figure 3A). The release of Hsp90/p23 was inversely correlated with the recruitment of NEMO. In contrast to Hsp90, Hsp70 was further recruited to activated PIDD in the cell free system (Figure 4a), which was also observed for CHIP (Supplementary Figure 3B).

To confirm this observation, we investigated the relationship between PIDD activation status and Hsp90 binding in HEK293T stably expressing PIDD, which exhibit constitutive

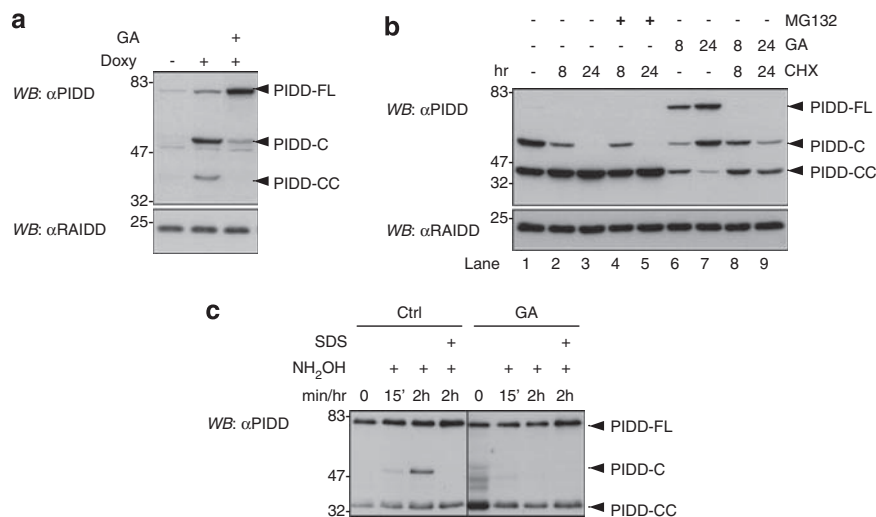


Figure 3 Hsp90 regulates the constitutive processing of PIDD. (a) Expression of PIDD in HEK293T Flp-In T-Rex cells was induced by 0.1 $\mu\text{g/ml}$ doxycycline in presence or absence of 1 μM GA for 8 h and analysed by western blotting. (b) HEK293T cells stably expressing PIDD were treated with CHX (10 $\mu\text{g/ml}$), GA (1 μM) or MG132 (10 μM) for the indicated times and the presence of PIDD fragments was monitored by western blotting. (c) Expression of PIDD F445H in HEK293T Flp-In T-Rex cells was induced by 0.1 $\mu\text{g/ml}$ doxycycline for 5 h in the presence or absence of GA (1 μM). PIDD was immunoprecipitated, eluted from the beads and treated with hydroxylamine to induce PIDD processing, which was analysed by western blotting. Blots were exposed simultaneously for the same amount of time. Molecular weight masses are indicated in kDa

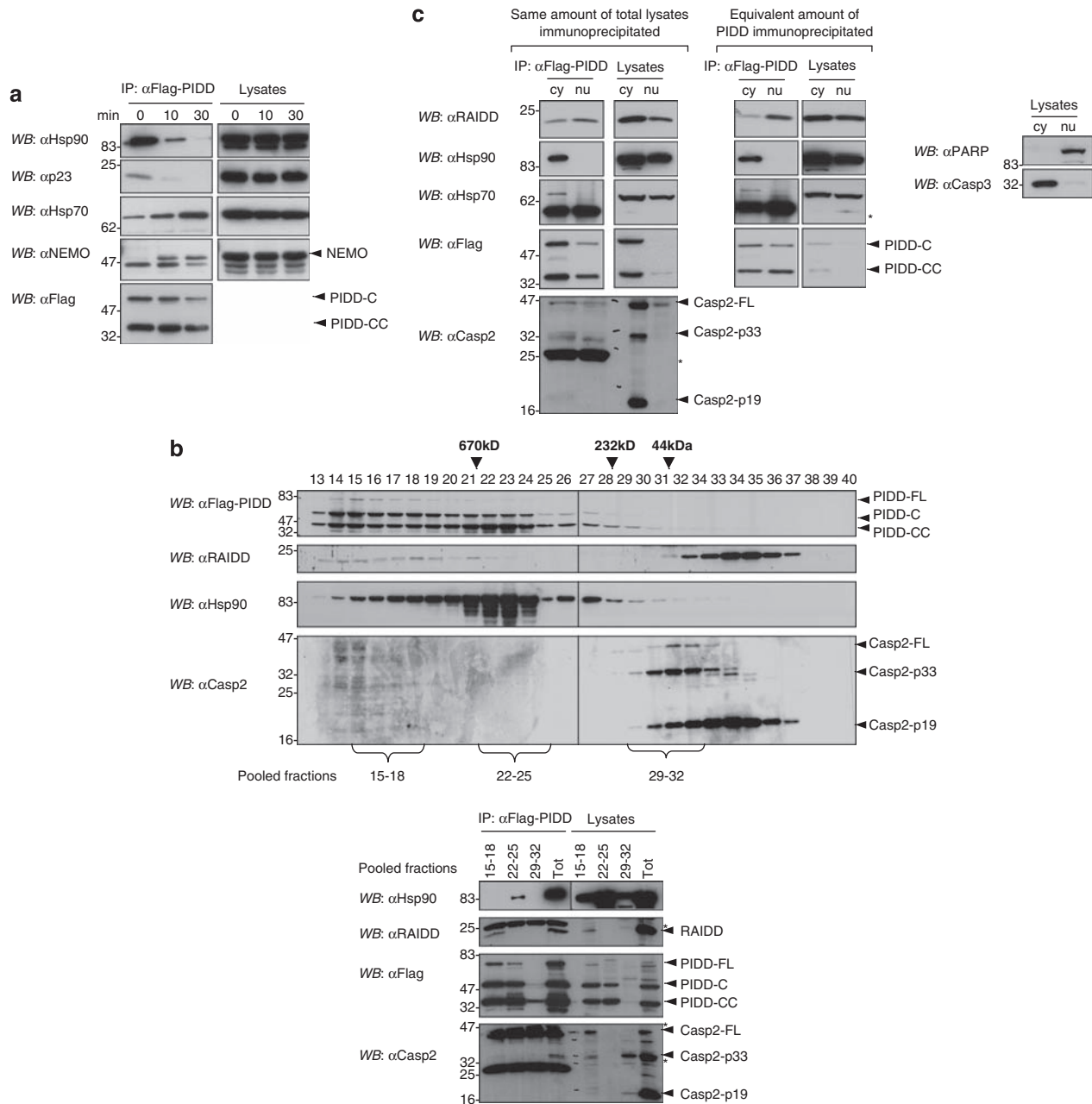


Figure 4 PIDDosome formation is associated with Hsp90 release. **(a)** A cell-free system experiment was performed on HEK293T cells stably expressing PIDD. Hypotonic extracts were incubated at 37°C for the indicated times and followed by anti-Flag immunoprecipitation and western blotting. **(b)** HEK293T cells stably expressing PIDD were lysed and loaded on a Superose-6B gel filtration column (0.5 ml/min). An aliquot of each fraction was loaded on a polyacrylamide gel and western blot analysis was performed. Anti-Flag immunoprecipitation was carried out on pooled fractions of the gel filtration column (15–18, 22–25 and 29–32) as well as on total lysates (before gel filtration; Tot) and analysed by western blotting. **(c)** Subcellular fractionation of HEK293T cells stably expressing PIDD was performed followed by two anti-Flag immunoprecipitations on the different fractions either using equivalent amount of lysate or equivalent levels of PIDD. Analysis was carried out by western blotting, and PARP and caspase-3 used as nuclear and cytoplasmic markers, respectively. Molecular weight masses are indicated in kDa and non-specific bands are marked by the asterisk

PIDDosome formation.⁴ In agreement with previous data,⁴ PIDD eluted in high and low molecular weight fractions in gel filtration experiments. In contrast, the majority of RAIDD eluted in fractions corresponding to its molecular weight (around 23 kDa) and only a small proportion eluted in high molecular weight fractions (Figure 4b). This latter pool corresponds to RAIDD complexed in the PIDDosome.

We suggest that more PIDD is found in high molecular weight fractions, because of PIDD being part of several different signalling complexes, not all of which contain RAIDD.^{5–7} IPs were carried out on different fractions of the gel filtration experiment. Fractions 15–18 were pooled and considered as the PIDDosome-enriched fraction (fractions in which RAIDD is present). Fractions 22–25 and fractions 29–32 were pooled

and considered as free PIDD-enriched fractions. The result shows that only non-complexed PIDD (fractions 22–25) interacted with Hsp90, whereas PIDD found in an active complex (fractions 15–18) was not found to associate with Hsp90 (Figure 4b). In fractions 29–32, containing PIDD-CC only fragments, these were not found to associate with Hsp90, in accordance with results shown in Figure 1e. PIDD translocates to the nucleus upon DNA damage,^{5,6} performing functional roles in DNA repair and cell cycle checkpoint maintenance (Logette E *et al*, personal communication).⁷ We therefore examined the localisation of PIDD-associated Hsp90 in HEK293T cells stably expressing PIDD. Cells were fractionated into cytoplasmic and nuclear samples and IPs of PIDD were performed with either equal amount of lysate or PIDD, as the levels differed between the two fractions (Figure 4c). We found that Hsp90 was exclusively associated with cytoplasmic and not nuclear PIDD. Moreover, despite the fact that the PIDDosome (as measured by the presence of RAIDD in the precipitates) can be detected in both the cytoplasm and the nucleus, it appears to be enriched in the nucleus, and a greater amount of RAIDD is found in complex with nuclear PIDD (Figure 4c, right panel). These data are in agreement with an activation-induced release of Hsp90 from PIDD and may suggest a role for Hsp90 in PIDD nuclear translocation following activation. The role of Hsp70 remains enigmatic, as it does not appear to bind nuclear PIDD at the basal level (Figure 4c) despite an overall increase in its association with PIDD following activation (Figure 4a).

Heat shock induces PIDD nuclear localisation and Hsp90 release. Owing to its identification as a p53-regulated gene and its death domain, PIDD has been extensively studied in response to genotoxic stress and in apoptotic induction.^{2,4–6,24} Little is known about other activators of the PIDDosome. However, a recent study by Green and colleagues^{25,26} identified caspase-2 as the initiator caspase activated during heat shock-induced apoptosis, this being dependent on RAIDD. We decided to investigate the PIDD-Hsp90 interaction during heat shock treatment. HEK293T cells stably expressing PIDD were subjected to heat shock at different temperatures. In a temperature-dependent manner, Hsp90/p23 was released from PIDD in our IP assay (Figure 5a). Partial release was already detected at 40°C incubation and substantial release of p23 and Hsp90 was observed at 42 and 43°C, respectively (Figure 5a).

Our previous data showing that PIDD translocates to the nucleus upon DNA damage^{5,6} and that active PIDDosomes are enriched in the nucleus in cells stably expressing PIDD (Figure 4c) may suggest a relocalisation of PIDD also during heat stress. We confirmed this hypothesis by confocal microscopy and subcellular fractionation. The results show that 45 min of heat stress induced a strong nuclear translocation of PIDD in both assays (Figure 5b and c and Supplementary Figure 4). PIDD translocation to the nucleus appeared to be very dynamic as PIDD nuclear staining decreased in a time-dependent manner when the cells were allowed to recover at 37°C after the heat shock (Figure 5b and c and Supplementary Figure 4). Moreover, we monitored the binding of Hsp90/p23 to PIDD by IP and observed that the interaction is lost during heat stress, but is recovered once the

temperature is reverted to 37°C. This points to a correlation between Hsp90/p23 binding, PIDD relocalisation and possible activation (Figure 5d). The interaction between Hsp70 and PIDD appeared less affected by heat shock treatment, mirroring the stability of this association during GA treatment and PIDD activation (Figures 1d, 4d, 5a and d).

Based on these results, further investigations to determine whether PIDD is actually activated during heat stress and how this affects a possible activation of caspase-2 would be of interest.

Hsp90 is required for PIDD functions. Besides a clear role in protein folding, molecular chaperones are also involved in the activation and function of their clients, as for example demonstrated by the requirement of Hsp90 during IKK activation.¹⁹ We examined the potential involvement of Hsp90 in PIDD functions. PIDD has been shown to induce NF- κ B in response to DNA damage through complex formation with RIP1 and NEMO, resulting in several modifications of NEMO (sumoylation, phosphorylation and ubiquitination).⁵ HEK293T wild-type cells were pretreated for 20 min with GA before NF- κ B induction by etoposide. Only short time points were studied to avoid the extensive degradation of PIDD observed after GA treatment (Figure 2a). Pretreatment with GA efficiently inhibited the phosphorylation of I κ B in response to etoposide, as shown in Figure 6a. PIDD levels remained constant during the course of the experiment and the activation of ATM, confirmed by its phosphorylation and the phosphorylation of p53, remained unchanged. Importantly, TNF α -induced NF- κ B activation was not inhibited by short GA pretreatment, demonstrating that RIP1 and IKK α , two Hsp90 clients involved in both TNF α - and etoposide-induced NF- κ B activation,^{18,19,27} remained functional in these settings (Figure 6a, right panel). Functional inhibition of NF- κ B was further confirmed by RT-PCR analysis of XIAP, a known NF- κ B responsive gene (Supplementary Figure 5). Furthermore, as observed for I κ B, NEMO phosphorylation and sumoylation were also affected by GA treatment, in contrast to p53 phosphorylation (Figure 6b).

Owing to the effects of GA seen on PIDD-mediated NF- κ B signalling, we investigated further consequences of this treatment on PIDDosome activity. As observed in cells stably expressing PIDD, induction of PIDD in HEK293T-Rex cells also activated caspase-2 as measured by p19 formation (Figure 6c and 6). GA treatment during PIDD induction resulted in the inhibition of caspase-2 activation as seen in Figure 6c, right panel. Caspase-2 and RAIDD recruitment to PIDD (formation of the PIDDosome) were reduced by the treatment (Figure 6c, left panel). In summary, disruption of the interaction between PIDD and Hsp90 (Figure 6c) by GA treatment affected the function of PIDD during NF- κ B signalling and PIDDosome-mediated caspase-2 activation.

Discussion

Heat shock proteins have become recognised as central players in cellular protein function, in particular their role in nuclear receptor biology has developed into an established model for chaperone activity.²⁸ Hsp90 was shown to be an essential component maintaining the naïve steroid receptor in

a hormone-binding competent conformation.²² Reminiscent of this chaperoning function, we have shown here that PIDD requires Hsp90 for an auto-processing competent conformation. Similar to PIDD, the nucleoporin Nup98 also exhibits strict structural requirements for HFS site auto-cleavage to occur;^{29,30} it would be interesting to establish whether Nup98 requires chaperones for proteolytic activity analogous to

PIDD. The structural details of this Hsp90-dependent conformation remain to be investigated, but the need for chaperones appears to be common among multi-domain proteins, which have to balance structural confinements with function. For example, LRR containing proteins, such as NLRs, have been shown to require Hsp90 and it has been suggested that chaperones might be able to support

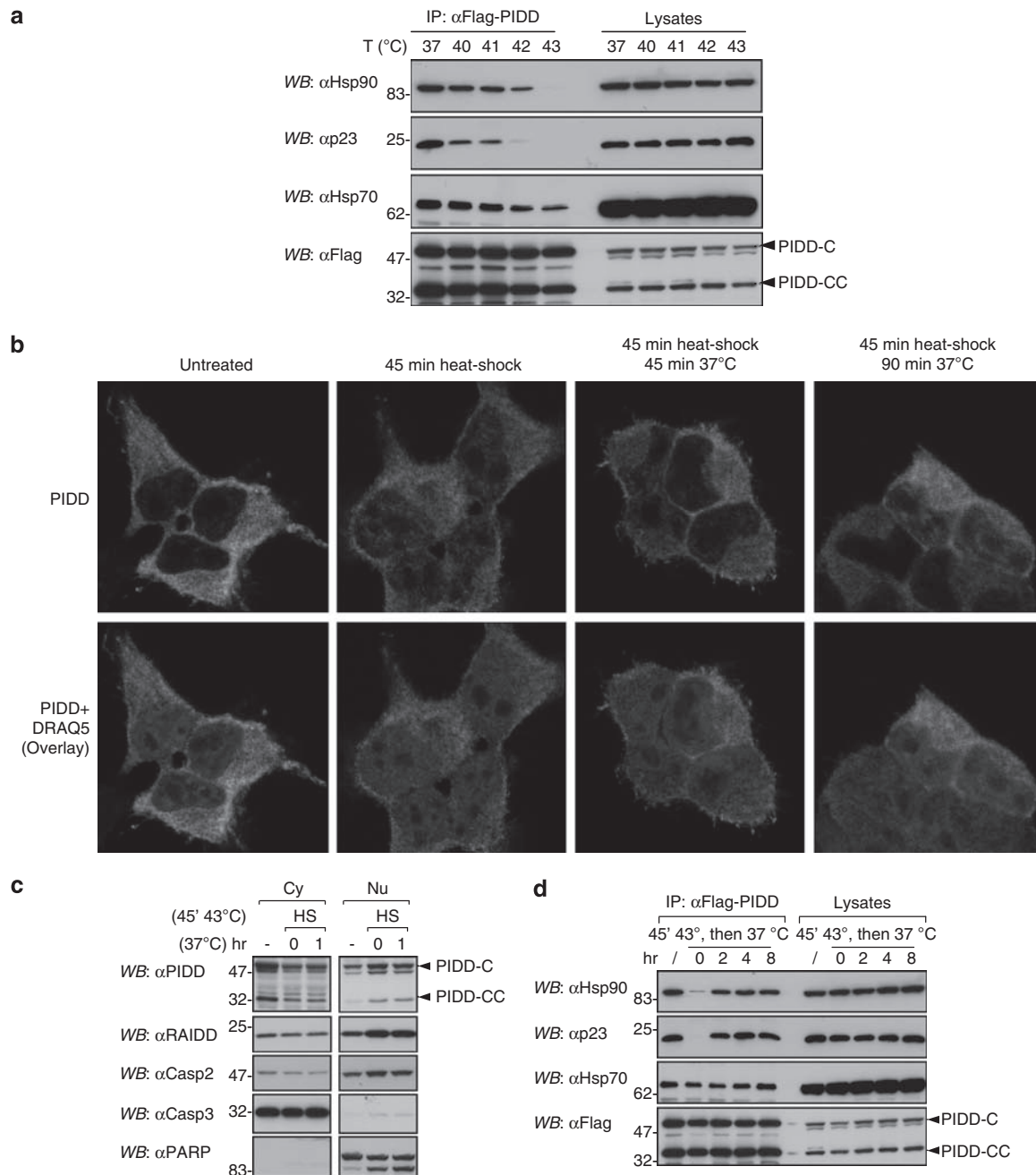


Figure 5 Heat shock induces PIDD/Hsp90 dissociation and PIDD nuclear translocation. **(a)** Heat shock treatments at the indicated temperatures were performed for 45 min on HEK293T cells stably expressing PIDD followed by anti-Flag immunoprecipitation of PIDD and western blotting. **(b)** Confocal staining of PIDD (top panels) or PIDD/DRAQ5 nuclear staining (bottom panels; overlay) in HEK293T cells stably expressing PIDD after a 45 min heat shock at 43 $^{\circ}$ C and subsequent recovery at 37 $^{\circ}$ C for 45 or 90 min, where indicated. **(c)** Subcellular fractionation of Jurkat wild-type cells after a 43 $^{\circ}$ C heat shock for 45 min and subsequent recovery at 37 $^{\circ}$ C (0 or 1 h). MG132 (10 μ M) was added, where indicated, and analysis was performed by western blotting. Caspase-3 and PARP were used as cytoplasmic and nuclear markers, respectively. **(d)** HEK293T cells stably expressing PIDD were subjected to a 43 $^{\circ}$ C heat shock for 45 min and were left to recover for the indicated time periods (0, 2, 4 or 8 h). Flag-tagged PIDD was immunoprecipitated and results obtained by western blotting. Molecular weight masses are indicated in kDa

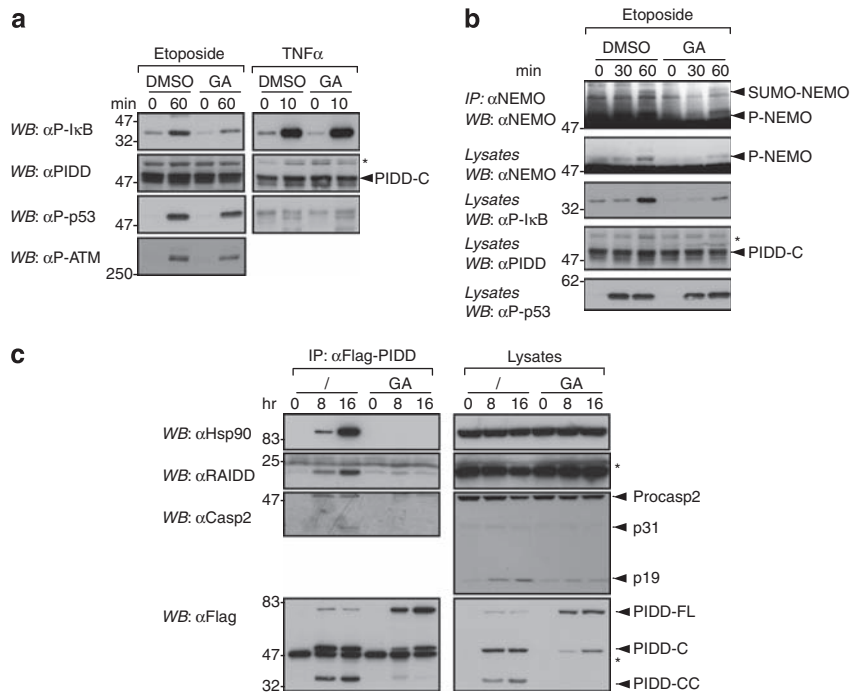


Figure 6 PIDD requires Hsp90 for its functional activity. (a) HEK293T wild-type cells were pretreated with 1 μ M GA for 20 min (etoposide treatment) or for 60 min (TNF α treatment) in presence of 10 μ M MG132. Etoposide (40 μ M) was added for 1 h and TNF α (10 ng/ml) for 10 min. Lysates were analysed by western blotting. (b) HEK293T cells were pretreated with 1 μ M GA for 20 min. Etoposide (40 μ M) was then added for the indicated times. An anti-NEMO immunoprecipitation was performed and analysed by western blotting. (c) PIDD expression was induced in HEK293T Flp-In Trex cells with 0.1 μ g/ml doxycycline for the indicated times in presence or absence of 1 μ M GA and an anti-Flag immunoprecipitation was carried out. Analysis was performed by western blotting. Molecular weight masses are indicated in kDa and non-specific bands are marked by the asterisk

less stable, but functionally advantageous LRRs in their clients.^{31,32} This concept might also hold true for the LRR-containing PIDD with its auto-catalytic challenges.

Structural studies on the related family member Unc5b demonstrated that under unstimulated conditions the ZU5-UPA-DD domain organisation, shared by PIDD, adopts a 'closed' conformation, impairing protein activity via an intramolecular auto-inhibitory mechanism. The authors propose that this conformation can be 'opened' through regulatory protein interactions or post-translational modifications.³³ It is tempting to speculate that Hsp90 may be involved in supporting a 'relaxed' PIDD structure (especially considering the hydrophobic nature of its core), allowing auto-cleavage and activation. This interaction could therefore serve as a regulatory step ensuring a dormant, but readily activated state of PIDD.

Molecular chaperones are thought to have functional consequences on protein complexes without being part of its final effector organisation.³⁴ In support of this hypothesis, we observed that activation of PIDD is associated with Hsp90 release and that nuclear PIDD enriched in signalling-competent PIDDosomes does no longer interact with Hsp90. In addition to the release of Hsp90 upon PIDD activation, we provide evidence that the Hsp90/PIDD interaction before activation is required for the function of PIDD in NF- κ B and caspase-2 activation. The results therefore demonstrate that although Hsp90 is required for PIDD auto-cleavage, stability and function, it is not a part of the final signalling complex, typical of chaperone proteins.

Interestingly, it was recently published that Hsp90 is an inhibitor of caspase-2 activation following heat shock treatment.²⁶ We found that Hsp90 is released from PIDD in response to heat stress. However, it remains to be seen whether PIDD is functionally activated by heat shock and whether this impacts on caspase-2, as its activation by heat shock is unclear at present.^{35,36} If heat shock can functionally activate PIDD, it is possible that Hsp90 may in this case serve as the sensor.

During our studies of these regulators, we observed an intriguing interaction of PIDD with Hsp70, which was increased during PIDD activation, but not found in the nucleus. For now the exact nature of this interaction remains unknown. Hsp70 is an important protein under normal and stress responsive cellular functioning, which greatly hinders studies involving its downregulation.³⁷ Moreover, an equivalent specific inhibitor, like GA for Hsp90, is still not available for Hsp70. However, we observed that, as with Hsp70, CHIP was recruited to PIDD upon activation *in vitro*. It has been reported that CHIP depends on Hsp70 for the degradation of some of its targets, such as the cystic fibrosis transmembrane conductance receptor.³⁸ Furthermore, CHIP is thought, apart from its functions in protein quality control, to also mark unwanted proteins for degradation, in particular in order to terminate signalling events.¹⁵ Therefore, the concomitant recruitment of Hsp70 and CHIP in response to PIDD activation may indicate a negative feedback loop on PIDD activity.

In addition, we observed differential interactions of Hsp90 and Hsp70 with the three isoforms of PIDD. These isoforms

have been described to exhibit different functions, for example, isoform-2 is thought to counteract isoform-1-driven proapoptotic effects.³⁹ Further studies are underway to investigate whether the differential interactions of Hsp90 and Hsp70 with the isoforms have functional consequences.

In conclusion, we propose that members of the molecular chaperone machinery, in particular Hsp90, have an important role as regulators of PIDD stability, its processing and its signalling function. It is evident that molecular chaperone offer critical support for complex-forming proteins like PIDD and that there remains many exciting questions on the role of chaperones in signalling pathways.

Materials and Methods

Cell culture and biological reagents. Human embryonal kidney 293T cells and HeLa cells were grown in DMEM + Glutamax (Invitrogen, Basel, Switzerland), supplemented with 10% fetal bovine serum (PAA Laboratories, Pasching, Austria) and 100 U/ml penicillin, 100 µg/ml streptomycin. Raji B cells, Ramos B cells and Jurkat T cells were grown in RPMI media (Life Technologies) with the same additives as DMEM. The generation of cells stably expressing PIDD was described elsewhere.⁴ Human recombinant TNF α was purchased from Adipogen (Epalinges, Switzerland), MG132 and etoposide were purchased from Alexis (Lausen, Switzerland). GA was obtained from InvivoGen (LabForce, Nunningen, Switzerland). Doxycycline, hydroxylamine, CHX and Flag-peptide were obtained from Sigma. Hygromycin was purchased from Invitrogen.

Expression vectors, siRNA and transfection. pCR3-PIDD-Flag and pMSCV-PIDD-Flag have been described previously.^{4,6} HEK293 Flp-In T-Rex cells expressing different constructs of PIDD were generated as described by the manufacturer (Invitrogen). Briefly, PIDD constructs were subcloned in pcDNA5/FRT vector (Invitrogen) and co-transfected with a Flp recombinase encoding-plasmid (pOG44, Invitrogen) in HEK293 Flp-In T-Rex cells. Clones were then selected with hygromycin. All PIDD constructs are C-terminally Flag-tagged. pcDNA3-CHIP was kindly provided by T R Hupp and A Zyliz. P23-specific siRNA and scrambled siRNA control were obtained from Qiagen (Qiagen AG, Switzerland), CHIP-specific siRNA was obtained from Ambion/Applied Biosystems (Invitrogen) and used at 50 nM for 48 h. CHIP transfections were carried out using the DNA-calcium phosphate method (for HEK293T cells; 2 µg of DNA) in 6 cm culture dishes for 48 h.

RT-PCR. HEK293T cells were pretreated where indicated with GA (1 µM) and etoposide (40 µM) was added for the indicated time periods. RNA was prepared with Trizol (Invitrogen) and 5 µg was used for a T-primed RT-PCR reaction (GE Healthcare, Glattburg, Switzerland). Specific primers were used to amplify GAPDH and XIAP (sense: 5'-TGGCAATATGGAGACTCAGC-3' and antisense: 5'-TGCACTTGTCACCAATACC-3').

In vitro PIDD processing. HEK293 Flp-In T-Rex cells expressing PIDD were induced for 5 h with 0.1 µg/ml doxycycline. The cells were lysed and an anti-Flag IP was performed. Immunoprecipitated PIDD was eluted with Flag peptides at 100 µg/ml in 50 mM Hepes pH 7.4, 150 mM NaCl. The eluates were then incubated in presence of hydroxylamine (NH₂OH) at room temperature.

IP, western blotting and antibodies. IP experiments were carried out in lysis buffer containing 1% NP-40, 20 mM Tris-HCl, pH 7.4, 250 mM NaCl (150 mM for endogenous IP), 1 mM EDTA, 5% glycerol and protease inhibitor mix. The ubiquitination assay was carried out in RIPA lysis buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 1 mM EDTA and protease inhibitor mix). Extracts were precleared with sepharose 6B beads (Sigma; 1 h, 4°C) and then incubated with anti-Flag (M2) agarose beads (Sigma) O/N at 4°C with continuous agitation or for endogenous IP with protein G beads (Amersham) together with anti-PIDD or anti-Flag (M2; Sigma) monoclonal antibody. The beads were washed three times with lysis buffer, eluted with Flag-peptide or directly resuspended in sample buffer, separated by SDS-PAGE, transferred to a Nitrocellulose membrane and analysed by immunoblotting. Antibodies used for western blotting were as follows: anti-phospho-I κ B, anti-phospho-ATM and anti-phospho-p53 (Cell Signalling, Denver, CO, USA), polyclonal anti-NEMO and anti-ubiquitin (Santa Cruz, Nunningen, Switzerland), monoclonal anti-NEMO, anti-RIP1

and anti-FADD (Transduction Labs), anti-p53 (DO-1), anti-caspase-2 11B4, rabbit anti-RAIDD and monoclonal anti-PIDD antibody (Anto-1) raised against PIDD-DD (776–910; Alexis), AL249, rabbit polyclonal antibody raised against PIDD (148–174; generated by Eurogentech, Liege, Belgium), anti-Hsp90 and anti-Hsp70 (Alexis), anti-p23 (Abcam, Cambridge, UK), anti-CHIP (Stressgen, Brussels, Belgium), anti-IKK α (Pharmingen, San Diego, CA, USA).

Confocal microscopy. HEK293T cells stably expressing PIDD were cultured on sterile glass coverslips in 6-well plates and fixed with a solution containing 1% paraformaldehyde, 2% glucose and 5 mM azide in PBS for 15 min at room temperature. Cells were permeabilised with 0.3% saponin for 10 min and treated with 2% normal goat serum/0.5% BSA/0.1% saponin as a blocking reagent. Flag antibody (M2, Sigma) was used at a dilution of 1/500 in 0.1% saponin/0.1%BSA/PBS; a secondary Alexa 488 anti-mouse IgG1 antibody (Invitrogen) was used at a dilution of 1/300 in 0.1% saponin/0.1% BSA/PBS. Cells were mounted in FluorSave (Calbiochem, Nottingham, UK), containing a 1/1000 dilution of DRAQ5 (Alexis) for nuclear counterstain. Images from immunostaining were collected by using a Zeiss inverted laser scanning confocal microscope LSM 510 with a 63 \times oil immersion objective.

Gel filtration. Cells were lysed in hypotonic buffer (20 mM HEPES-KOH, 10 mM KCl, 1 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM DTT (pH 7.5)) and fractionated using a FPLC protein purification system on a Superose-6B column (Amersham Life Science) at 4°C. The column was equilibrated with the lysis buffer, and lysates (2 mg) were applied and eluted from the column at a flow rate of 0.5 ml/min and 500 µl fractions were collected. The column was calibrated with Amersham gel filtration standards containing bovine thyroglobulin (670 kDa), catalase (232 kDa) and chicken ovalbumin (44 kDa).

Subcellular fractionation. Cytoplasmic and nuclear fractions were prepared according to a previously published protocol.⁴⁰ Briefly, cells were lysed in a cytosolic lysis buffer containing 10 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 300 mM sucrose, 0.5% NP-40, 10 mM KCl, supplemented with DTT and protease inhibitor mix. Nuclei were pelleted by a short centrifugation and lysed in a nuclear buffer containing 20 mM HEPES pH 7.9, 100 mM NaCl, 0.2 mM EDTA, 20% glycerol, 100 mM KCl, supplemented with DTT and protease inhibitor mix. Nuclear pellets were subjected to three freeze/thaw cycles, sonicated and centrifuged to obtain a soluble nuclear fraction.

Heat shock. Heat shock was performed at 43°C in a water bath for 45 min and the cells were allowed to recover at 37°C for the indicated periods of time. Cells were lysed and where indicated IP was performed.

In vitro PIDD activation. Hypotonic lysates (lysis buffer: 20 mM HEPES-KOH, 10 mM KCl, 1 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM DTT (pH 7.5)) were prepared from HEK293T cells stably expressing Flag-tagged PIDD as previously described⁴ and incubated at 37°C for the indicated times. For further IP analysis following incubation an equivalent volume of lysis buffer containing 0.1% NP-40 and 300 mM NaCl was added.

Protein interaction screens. The yeast-two hybrid screen was performed by hybridogenics using the amino acids 1–319 of PIDD against a human library. The proteomics screens were carried out on subcellular fractions of HEK293T cells stably expressing PIDD. Flag-tagged PIDD was immunoprecipitated and samples run on polyacrylamide gels, which were subsequently stained by Coomassie. Bands were excised, trypsin digested and analysed by shotgun LC-MS/MS.

Conflict of interest

The authors declare no conflict of interest.

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Author contributions

AT, MJE and JT designed the research, analysed the data and wrote the paper; AT, MJE, SL, EL, SJ, BJ and MQ performed the research.

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Supplementary Information accompanies the paper on Cell Death and Differentiation website (<http://www.nature.com/cdd>)