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News and Commentary

Ubiquitylation in apoptosis: DIAP1's (N-)en(d)igma

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A Piece of Advice…

'…don't waste your time on genetics…They keep tormenting fruit flies, but it's us biochemists who will produce the understanding that really matters.'[1]
…was the somewhat unsound advice given to, and thankfully ignored by, Alexander Varshavsky in his formative undergraduate days. As through a powerful combination of both fields, our understanding of a cell's essential intracellular proteolytic system has made huge impacts in biology and now such findings are beginning to be translated into medical applications.[2]

Primarily, in the *Drosophila* field, apoptosis represents one of the current areas with a 'buzz' of ubiquitin-related excitement to it. In particular, focusing on the role of *Drosophila* Inhibitor of APoptosis protein 1 (DIAP1), a protein that bears a carboxy-terminal RING-finger domain, and its role in, unsurprisingly, inhibiting apoptosis. Although still controversial, Inhibitor of Apoptosis proteins' (IAPs') links to the ubiquitin-proteasome system (UPS) are both considerable and varied. Indeed, the mechanisms involved and the true impact of IAP-associated ubiquitylation are only just emerging.

All of the major components of the UPS are present in *Drosophila*, albeit, in comparison with mice and humans, at reduced numbers across each class. Taking E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme), E3 (ubiquitin-protein ligase) and DUB (deubiquitylating enzyme) (see Table 1) representation as a whole, *Drosophila* harbours 61 and 57% less components than human and mouse, respectively, but 57% more than budding yeast. Therefore *Drosophila* represents an ideal organism to act as a stepping-stone between the extensive findings in the unicellular context of yeast and towards translating those findings into a multicellular context. A reduced complexity helps to bypass redundancy or compensation and, in combination with the power of *Drosophila* genetics, as well as their accessibility to molecular biology and biochemistry, should facilitate further mechanistic discoveries.

IAPs

As in many other fields viruses have, and still continue, to provide insights into the importance and mechanisms of both pro- and antiapoptotic proteins and their pathways. The founder members of a family of proteins called (IAPs), were first discovered in the baculoviruses *Cydia pomonella* granulovirus and *Orgyia pseudotsugata* M nucleopolyhedrovirus and implicated in the viruses' ability to inhibit host-cell apoptosis.[3] And representatives of this protein family are present in worms through to mammals. Members are defined by the presence of the Baculoviral Inhibitory Repeats domain that, in combination with its flanking region, is important for mediating protein–protein interactions.[4]

Caspases

One such group of IAP-interacting proteins are caspases. These aspartate-directed cysteine proteases provide the proteolytic backbone of programmed cell death.[5,6] In *Drosophila*, DIAP1's ability to bind and inhibit caspases,[7] enables it to act as one of the last lines of defence before apoptotic Armageddon.

Caspases can be grouped into either initiator or effector caspases, both of which can be bound and regulated by DIAP1.[7] Effector caspases represent the apoptotic workhorses, actively cleaving a selective set of substrates involved in a diverse array of cellular functions – promoting DNA fragmentation and general collapse of cellular architecture.[8] They are activated through proteolytic cleavage by initiator caspases, which in turn are activated upon recruitment into macro-molecular protein complexes.[6] Indispensable to DIAP1's ability to inhibit both classes of caspases are its two BIR domains.[7] While the BIR1 region binds to effector caspases DCP-1 and drICE, it is the BIR2 domain that binds the initiator-caspase, DRONC.

Intriguingly, caspase-mediated cleavage of effector-caspases not only activates these caspases, but simultaneously exposes them to inhibition by DIAP1.[9] Removal of the caspases' amino-terminal prodomain creates a new amino-terminus which bears an alanine residue; and exposure of this small, hydrophobic amino acid is essential for the BIR1:caspase interaction.[9] Similarly, DRONC's interaction with the BIR2 domain utilises a short, but internal DRONC sequence that binds to an analogous groove in the BIR2.[10]

DIAP1-mediated inhibition of caspases through binding alone remains a simple and viable model of regulation. Indeed, some IAPs, *in vitro*, can inhibit effector-caspases' proteolytic activity towards certain substrates.[3] However, DIAP1, unlike the mammalian X-linked IAP (XIAP), fails to act as a *bona fide* enzyme inhibitor, instead regulating, but not ablating, the caspases' catalytic potential. Consistently, DIAP1 lacks homology to the caspase-binding residues of

Table 1 Some ubiquitylation-associated definitions

N-end Rule: A relation between the metabolic stability of a protein and the identity of its amino-terminal residue
N-degron: For a degradation signal to be termed an N-degron, it is necessary and sufficient that it contains a substrate's initial or acquired N-terminal residue whose recognition by the targeting machinery is essential for the activity of this degron
Pre-N-Degron: Features of a protein that are necessary and sufficient for the formation of an N-Degron
E1 (ubiquitin-activating enzyme): An enzyme that activates ubiquitin for the array of downstream-conjugating enzymes
E2 (ubiquitin-conjugating enzyme): An enzyme that transiently carries an activated ubiquitin as a thiol ester and acts as an intermediate, before transfer of ubiquitin to a substrate
E3 or ubiquitin–protein ligase: A protein that binds directly, or indirectly, specific protein substrates and promotes the transfer of ubiquitin, directly or indirectly, from a thiol-ester intermediate (such as from an E2) to lysine residues on proteins or polyubiquitin chains
N-Recognin: A part of the targeting machinery that functions as an E3 ubiquitin–protein ligase and recognises an N-degron
DUB: Deubiquitylating enzyme that specifically cleaves ubiquitin-linked molecules after the terminal carbonyl of the last residue of ubiquitin – glycine

XIAP, suggesting that these two IAPs neutralise caspases differently.

Surprisingly, DCP-1 bound to DIAP1 remains catalytically active towards DIAP1 – as following binding it still can cleave DIAP1.[9] It thus raises the question of how DIAP1 might further deal with a bound, but catalytically competent caspase? In fact, a growing body of evidence suggests that caspase-binding alone, although necessary, is not sufficient for caspase regulation *in vivo*.

Belt, but no Braces?

In its role to regulate caspase activity, DIAP1-associated ubiquitylation may supply an answer to the question of how DIAP1 deals with a bound but active caspase – providing DIAP1 with, in addition to caspase binding, an added string to its antiapoptotic bow. Described below are two putative mechanisms through which DIAP1 may regulate caspases and therefore apoptosis.

The N-end Rule

Following effector caspase binding, DIAP1 is efficiently cleaved at its amino-terminus, whereby it loses its first 20 amino-acid residues,[11] a stretch that does not harbour any recognised motifs and is not required for caspase binding.[9,12] Nevertheless, removal of this region through caspase-mediated cleavage radically changes DIAP1's properties. Here it is important to remember that intracellular proteolysis is a common and important mode of post-translational modification. As exemplified by the generation of peptide hormones, antigens and the activation of proteases, such as caspases. Therefore, intracellular proteolysis can provide a very specific mechanism of activating and/or inactivating a protein's properties. Additionally, proteolysis can also influence the metabolic stability of proteins. Hence, proteolysis can affect a protein's function through regulating both its activity and/or its overall steady-state level.

In 1986, Alexander Varshavsky's group provided an important link between a substrates' intracellular proteolysis and its subsequent degradation.[13] Establishing a genetic system in yeast, known as the ubiquitin fusion technique (UFT), Bachmair, Finley and Varshavsky identified both the first protein degradation signal as well as the first set of rules that specifies how certain proteins are recognised for ubiquitylation and subsequent proteasomal degradation.

Using the UFT they identified a relationship between the amino-terminal amino acid of a protein and its metabolic stability – dubbed the 'N-end Rule'.[14] This simple, but elegant rule proved to hold true, with some minor differences, across all known eukaryotes. Alternative protein amino-termini can be generated through various proteolytic mechanisms, including the action of methionine amino-peptidases and caspases. Such actions remove the universally stable N-end rule residue, methionine, and expose a new residue of the N-end rule. To date, thanks to the identification of the components of this pathway, the N-end rule and its associated degradation signal, called an N-degron (Table 1), remains one of the best-understood ubiquitin-dependent degradation systems. Accordingly, for this discovery and for his contributions to understanding a physiological role for the UPS, Alexander Varshavsky has been championed for a future Nobel prize in Physiology or Medicine.[15]

Within the metazoan N-end rule, all destabilising amino acids can be classified into three groups, albeit with some variation across species, namely, type-1, type-2 and type-3 residues.[14] These classifications can also be loosely defined by their chemical properties (basic, bulky or small), which in turn govern which of the binding pockets they interact with on the N-end rule's E3. Hence, the amino-terminal amino acid exposed on the substrate directs the interaction with the N-end rule's E3.

In the case of the cleaved form of DIAP1, an amino-terminal asparagine represents a tertiary destabilising residue, which after deamidation to aspartate and subsequent arginylation binds to the N-end rule's E3, UBR1 (Figure 1a).[14] Consequently, DIAP1, through UBR1's recruitment of the N-end rule's associated E2, UBCD2, is then presumably ubiquitylated and targeted for degradation. In other words, caspase-mediated cleavage of DIAP1 triggers the recruitment of an E3 and its associated E2 to the DIAP1:caspase complex. And the outcome of this E2-recruitment is the conversion of DIAP1 into a highly unstable protein.[11] In contrast, a noncleavable form of DIAP1, which is unable to generate the asparagine-bearing DIAP1, is relatively stable. Surprisingly, such a stable, noncleavable DIAP1 displays a reduced ability to inhibit apoptosis in comparison to the wild-type form.[11,16] Therefore, rather counterintuitively, DIAP1 instability seems important for its antiapoptotic function. As with the proteolytic activation of effector-caspases described above, proteolysis does not necessarily lead to loss of function, but can actually provide a protein with an alter ego.

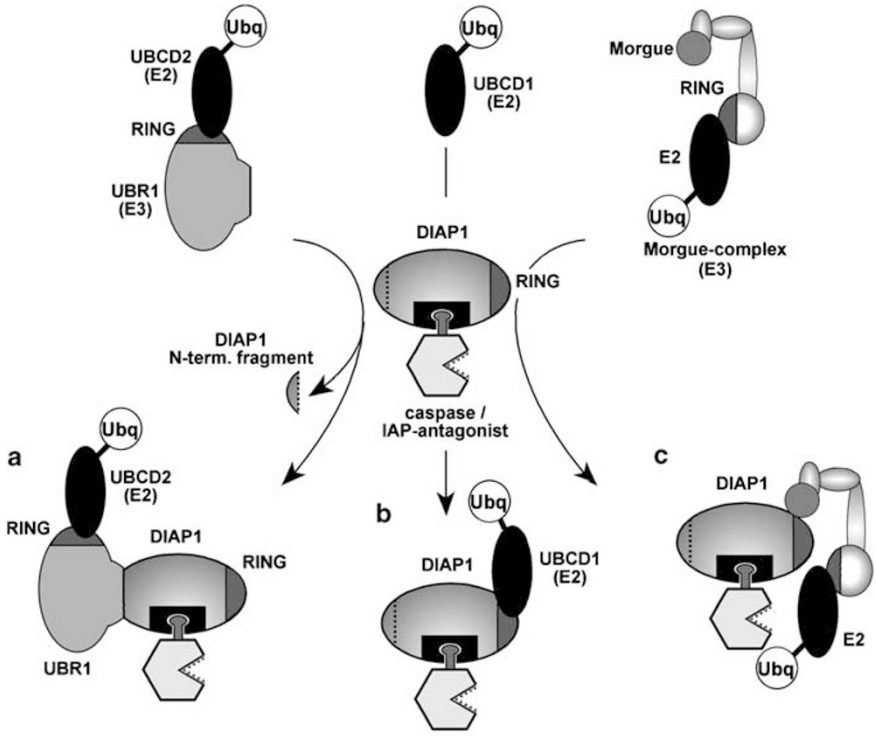

Figure 1 DIAP1, through three separate RING-mediated interactions, could recruit three different E2 ubiquitin-conjugating enzymes. E2 recruitment can lead to the ubiquitylation of either DIAP1 and/or DIAP1-BIR-associated proteins, such as caspases and IAP antagonists. (**a**) Caspase-mediated cleavage of DIAP1 leads to the recruitment of the N-end rule E3, UBR1 and its associated E2, UBCD2. (**b**) DIAP1's RING domain directly recruits the E2, UBCD1. (**c**) A putative Morgue containing SCF E3-complex could recruit an additional E2 to DIAP1. Ubq denotes a ubiquitin moiety. E2s are shown as black ovals

Hence, DIAP1 may sense caspase activity through caspase-mediated cleavage, which instead of opposing, actually may activate DIAP1's antiapoptotic ability. One possible mechanistic explanation could be through the codegradation of both cleaved DIAP1 along with the associated active caspase. Such a model provides a stoichiometric method of coregulating the levels of two different proteins. Recruitment of UBR1 and degradation via ubiquitylation therefore provides a powerful mechanism for how DIAP1 can absolutely inhibit caspase activity – as degradation is forever. Additionally, the formation of a DIAP1:UBR1 complex can be viewed as a heterodimeric E3, whereby DIAP1 acts as the substrate linker, recruiting effector caspases to the N-end rule E3-associated E2, UBCD2 (Figure 1a).

Similar observations of caspase-mediated DIAP1 instability are reported with a separate DRONC-mediated cleavage site in DIAP1.[16] Cleavage at this site generates another highly unstable DIAP1 molecule. Similarly to what is described for effector caspases, it seems that the instability conferred by DRONC-mediated DIAP1 cleavage, is also important for activating DIAP1's antiapoptotic ability. Although the participation of the N-end rule has not formally been shown, such a cleavage exposes threonine, a type 3 destabilising residue.[14]

Hence, DIAP1's E3 activity may be activated through caspase-mediated cleavage – ultimately resulting in the coordinated destruction of both the IAP and the active caspases. In this case, DIAP1 upon cleavage, and only upon cleavage, exposes a new amino-termini, which recruits the N-end Rule E3 degradation system and links it up with the active caspase responsible (Figure 1a).

Controversy remains over the need for an additional event downstream of caspase binding, such as the coordinated destruction model proposed above, for DIAP1's antiapoptotic function. Especially as overexpression, to unphysiologically high levels, of various ubiquitylation-impaired DIAP1 mutants still provide some degree of apoptotic protection,[17] albeit significantly reduced in comparison with wild-type DIAP1.[11,18] Overall, these findings support the idea that binding alone can impair caspase function.

A Role for DIAP1's RING

However, additional evidence supporting a second function of DIAP1 in apoptotic regulation resides within DIAP1's carboxyl-terminal, classical RING-finger domain. A highly conserved domain that is present in over 400 functionally diverse proteins and is defined by a pattern of conserved cysteine and histidine residues that coordinate two zinc atoms.[19] The zinc coordination is essential for the correct folding of this domain that is, functionally, heavily implicated in ubiquitylation.

In fact, RING-finger domains, in addition to associated motifs, are recognised as protein folds that bind to E2 ubiquitin conjugating enzymes.[19] Indeed, the *Drosophila* E2, UBCD1, binds directly to DIAP1 (Figure 1b).[20] Many IAPs wield a RING-finger domain[21] and therefore could potentially function

as molecular scaffolds, bridging an E2 to substrates. Such properties fit in well with the quite loose definition of an E3: a protein that binds directly, or indirectly, specific protein substrates and promotes the transfer of ubiquitin, directly or indirectly, from a thiol-ester intermediate (such as an E2-ubiquitin intermediate) to amide linkages with proteins or polyubiquitin chains.[22] Of note, the RING-finger domain has no intrinsic ubiquitylation activity, other than mechanistically to bind and recruit an E2 into the vicinity of a substrate.[23] Subsequently promoting the ubiquitylation of that substrate.

Studies on the viral IAP proteins revealed that their RING-finger domains are essential for suppressing host cell apoptosis.[3] Analogously, this also seems to be the case for DIAP1. Genetic screens for modifiers of proapoptotic proteins exposed DIAP1's RING finger as being essential for cell viability.[3] The identified RING mutants (e.g., DIAP1$^{21\text{-}4}$) are all predicted to abolish E2 binding and accordingly, fail to ubiquitylate the initiator caspase, DRONC.[10,24] Importantly, DIAP1 RING mutants show no defect in DRONC binding, but completely fail to regulate this caspase *in vivo*.[25] Clearly, *in vivo*, a DIAP1 protein still capable of binding caspases, but unable to promote their ubiquitylation, fails to regulate apoptosis.

A growing body of evidence suggests that DIAP1-mediated DRONC ubiquitylation leads to its degradation (Figure 1b), representing an absolute method of negatively regulating DRONC activity. Most strikingly, DRONC levels in a DIAP1$^{21\text{–}4}$ background *in vivo* seem elevated,[26] supporting the idea that DIAP1-mediated DRONC ubiquitylation, and its subsequent degradation, is impaired. And it is this impairment that explains, in part, the DIAP$^{21\text{–}4}$ apoptotic phenotype.

Mutually Assured Destruction?

Owing to the presence of its RING domain, DIAP1 has undergone numerous *in vitro* and *in vivo* ubiquitylation assays.[27] Addressing both its role as a potential E3 towards its protein-interaction partners as well as acting as a substrate for ubiquitylation itself. Many E3s, in addition to mediating ubiquitylation of other proteins, seem to undergo ubiquitylation themselves – and DIAP1 is no exception to this. Within the subclass of RING E3s, the formation of a physically linked ubiquitin-E3 intermediate is unnecessary.[22] Hence, RING-bearing E3s do not need to be ubiquitylated in order to promote ubiquitylation of their target substrates. So, why is DIAP1 being ubiquitylated?

It is important to remember that it is the E2 that mediates E3 ubiquitylation. The observation that E3s are ubiquitylated is often described as 'autoubiquitylation'. However, 'autoubiquitylation' is not an intrinsic ability of the E3 itself, but really reflects RING-directed, E2-mediated ubiquitylation of a receptor lysine on the E3. Hence, in this respect the E2 ubiquitylates the E3 rather than a *bona fide* substrate (a protein bound to the E3). E3 ubiquitylation may either simply represent an occupational hazard of dealing with a ubiquitin-conjugating enzyme, or perhaps, may signify an important regulatory event. It remains possible, however, that the RING domain, under certain situations, can act as a degradation signal whereby only the RING-bearing protein is targeted for degradation. Such a mechanism could ensure homeostatic regulation of the steady-state levels of a RING-bearing protein. Yet, as mentioned above in the case of the N-end rule, protein instability may actually provide a protein with some functionality. Indeed, DIAP1 RING mutants display increased protein stability levels but actually exhibit loss of function phenotypes.

In a situation where DIAP1 is caspase-binding-competent, but ubiquitylation deficient, it can no longer effectively suppress apoptosis. These observations, like those seen with the DIAP1 N-end rule mutants, argue against the simple idea that DIAP1 caspase binding alone is sufficient to efficiently inhibit apoptosis. In such a scenario, perhaps DIAP1's instability, either via the N-end rule or the RING domain, reflects part of its normal job in apoptotic regulation; constantly dealing with unwanted caspase activity through ubiquitin-mediated degradation of both itself and the associated active caspase.

In this respect, DIAP1's instability may act as an indicator for when an E3 is active towards its substrate. However, upon initiation of apoptosis, insurmountable levels of active caspases results in the concomitant destruction of DIAP1 – in its valiant attempt to remove caspase activity – quite literally, working itself to death. Clearly, the protein levels of DIAP1 will be absolutely critical in establishing a threshold level for apoptosis. Lowering this threshold by inhibiting DIAP1's *de novo* protein synthesis induces cells to undergo rapid, caspase-dependent apoptosis.[27] Emphasising that cells are addicted to DIAP1 and are therefore acutely sensitive to DIAP1 protein levels. On a simple level, either too high, or too low, a level of DIAP1 can represent an unwanted cellular situation: too low and cells will no longer be able to restrain any unwanted caspase activity and undergo apoptosis; while too high, and a cell will be unable to commit apoptosis. Both scenarios highlight the importance of adjusting DIAP1's levels to suit certain cellular situations.

The highly labile nature of proteins is frequently a trademark of key regulatory molecules. Such proteins exhibit very short half-lives to allow rapid adjustments of their levels. Why DIAP1 should be so unstable remains debatable, but it seems energetically inefficient to rapidly turnover proteins without a good reason. A plausible explanation, as suggested above, is that DIAP1's degradation is part of normal cellular activity in response to active caspases.

We shall Overcome

While IAPs function to suppress the activation and/or activity of caspases in healthy cells, this IAP-mediated inhibition needs to be overcome in cells fated to die. A group of proapoptotic proteins, the IAP-antagonists,[4] provide such a role acting, in part, by downregulating DIAP1 protein levels. These proapoptotic, BIR-interacting proteins can, through competitive binding with caspases for DIAP1's BIR domains, prevent and/or displace caspases from DIAP1 association.[4] As with caspases, an exposed amino-terminal alanine on the IAP-antagonists is absolutely essential for binding to DIAP1's BIR domains.

On an elementary level, IAP-antagonists through binding alone can inhibit caspase:DIAP1 association and therefore remove, both binding and ubiquitylation-associated,

DIAP1-mediated caspase inhibition. However, as with the caspase:DIAP1 association, the relationship between DIAP1 and IAP-antagonists may incorporate more than just binding alone. A number of groups have demonstrated both IAP-antagonist-mediated enhancement of DIAP1's ubiquitylation and its instability.[27] Therefore, IAP-antagonists not only displace caspases from DIAP1-mediated inhibition, but can also remove DIAP1 through its degradation. This belt-and-braces, two-pronged, approach would remove, through both caspase displacement and DIAP1 degradation, DIAP1's apoptotic inhibition and ensure the cells' final commitment to apoptosis.

In a further twist, other groups reported that IAPs can promote the ubiquitylation of IAP-antagonists.[28,29] Suggesting that DIAP1 may promote the degradation of these proteins, providing a regulatory battle between two proteins hell-bent on trying to destroy each other.

The observation that DIAP1's ability to bind and degrade IAP-antagonists is strictly dependent on an amino-terminally exposed alanine, raises the possibility that DIAP1 may represent an E3 for the alanine-bearing branch of the N-end rule. Although little is known about this branch, DIAP1, with its strict binding requirements and the presence of a RING domain, qualifies it for such a role.

A (st)RING of E2s

It appears that DIAP1 can associate with multiple E2s (Figure 1); through its RING finger with UBCD1,[20] or via UBR1 with UBCD2. Moreover, DIAP1 can also interact with an F-box protein, Morgue,[27] which may be part of a multisubunit, RING-containing, SCF E3-complex.[30] Such an interaction provides yet another indirect method for recruiting an E2 to DIAP1 (Figure 1c). Therefore, there may be a scenario where separate RING-wielding systems (UBR1, SCF E3-complex and DIAP1), and therefore RING-associated E2s, are all anchored into DIAP1. These three systems may work in isolation, or perhaps cooperatively, determining both the type and/or the degree of protein ubiquitylation.

As the list of proteins associated with DIAP1 steadily grows, an obvious comparison can be seen with MDM2 and the host of proteins that bind and affect its role as an E3.[31] Both MDM2 and DIAP1 carry RING-finger domains, a domain that is a common component of molecular scaffolds, perhaps highlighting the requirement for high-order architecture and the involvement of multiple protein partners. Indeed, MDM2 is capable of also associating with another RING-bearing protein MDMX.[31]

Conclusions

It seems that ubiquitylation is used both as a means of promoting and inhibiting apoptosis. DIAP1 can both negatively regulate proapoptotic molecules, as well as being negatively regulated itself, through ubiquitylation. Nearly all of the DIAP1-interacting proteins reported here, including DIAP1 itself, are either ubiquitylated and/or somehow associated with the UPS itself. Although most reported DIAP1-associated ubiquitylation events have been linked to degradation, it is highly important to point out that not all ubiquitylation events result in degradation. As chain length, as well as lysine linkage within the chain, can radically affect the modified proteins outcome.[32]

On top of the situations described, other post-translational modifications are likely to impact on DIAP1-associated ubiquitylation events. For example, recent work has implicated a role for the MST1/2 family kinase, Hippo, in promoting DIAP1 phosphorylation and degradation.[33]

It still remains unclear whether DIAP1 instability is a strict requirement for its function as an E3 or simply reflects regulation of it steady-state levels. Thus, the RING may be a dual-function domain: (1) to regulate a RING-bearing protein's stability – thereby acting as a degradation signal, or (2) to facilitate the ubiquitylation of E3-associated substrates. Switching between these two states could be influenced by a myriad of events. Alternatively, the true situation may be somewhere in-between-with the two outcomes being inseparable and acting in concert, perhaps reflecting a form of coregulation between an E3 and its substrate. Regardless, DIAP1's initial dalliance with ubiquitylation may well turn into a rather vicious melee.

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