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Pallbearer and friends: lending a hand in the clearance of apoptotic cells

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Abstract

Engulfment and prompt removal of apoptotic cells occurs from embryogenesis throughout the lifespan of multi-cellular organisms. A new player, Pallbearer, has recently been identified in *Drosophila* as being important for efficient engulfment by macrophages. Pallbearer is a component of the SCF E3 ubiquitin ligase complex involved in ubiquitylation of proteins targeted for proteasomal degradation. This work provides the first link between the cellular processes of ubiquitylation/proteasomal degradation and the ability to efficiently clear apoptotic cells.

The efficient engulfment and removal of apoptotic cells is important for tissue homeostasis and immunological self-tolerance ¹. Such engulfment is carried out by competent neighboring cells or by professional phagocytes, such as macrophages and immature dendritic cells. In recent years, the molecular underpinnings of this process have begun to be revealed through studies in the model organisms *C. elegans* and *Drosophila*, along with molecular and biochemical studies in mammalian cells and via knockout mice ^{2, 3}. Three key steps in the phagocytic removal of apoptotic cells are recognition, internalization and degradation. Apoptotic cells display several “eat-me” markers that serve as molecular cues for phagocyte recognition and to initiate engulfment via receptors on the phagocyte. The most prominent of these “eat-me” signals is the exposure of phosphatidylserine (PS) on the outer leaflet of the plasma membrane ⁴. Multiple modes of PS recognition have been defined, including the recent identification of direct PS-binding receptors Bai1, Tim4 and Stabilin-2 ^{5–7}. Upon encountering the apoptotic target, rearrangement of the actin cytoskeleton occurs in the phagocyte, driven primarily by the Rho GTPase Rac ⁸. This leads to the formation of an actin-rich phagocytic cup that envelops the apoptotic cell. After internalization, the corpse proceeds through the phagolysosomal pathway where the remnants of the apoptotic cell are degraded and processed. Although the degradative step remains poorly understood, this process is clearly important for antigen presentation and protein recycling and may impact the efficiency of corpse engulfment ^{9, 10}.

The post-translational modification of proteins by ubiquitylation has a well-documented role in targeting proteins for proteasomal degradation and regulation of numerous cellular processes including protein transport, cell cycle progression, DNA repair and inflammation ^{11–13}. Ubiquitylation is carried out through a series of enzymatic steps that results in the covalent attachment of the 8 kDa ubiquitin (Ub) protein to lysine residues on substrate proteins. The Ub-activating enzyme (E1) transfers Ub to Ub-conjugating enzymes (E2). The Ub-ligases (E3) bind to substrate proteins and promote transfer of the Ub from E2 to specific lysine residues on the target protein. Substrate specificity for ubiquitylation is determined by

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the E3 ligases, which specifically interact with proteins based on sequence determinants and/or post-translational modifications such as phosphorylation. There are two types of E3 Ub ligases: HECT (*homologous to E6-AP carboxyl terminus*) and RING (*really interesting gene*), which differ in how they promote ubiquitylation. HECT E3's serve as an intermediate acceptor of Ub from E2 prior to transfer of the Ub to the substrate. RING E3 complexes bridge Ub-conjugated E2 and the substrate allowing for transfer of Ub from E2 to substrate.

Among the RING E3 ligases, the best characterized is the SCF complex, composed of Skp1, Cul1, an F-box protein (hence the name SCF) and Roc1. Cul1 tethers the Roc1-Ub-conjugated E2 complex to the Skp1-F-box-substrate complex (Figure 1). Because the F-box protein interacts with the substrate, it is the key determinant in substrate specificity and recruitment to the SCF ubiquitylation machinery. The *Drosophila* genome contains at least 33 F-box proteins, some of which have been characterized, including Slimb, Ago and Morgue which regulate a broad range of basic cellular operations by targeting proteins for degradation¹⁴. However, very little is known about the role of F-box proteins or even ubiquitylation as it relates to phagocytosis of apoptotic cells.

Recently Nathalie Franc and colleagues have demonstrated for the first time a role for the SCF-ubiquitylation complex in the uptake of apoptotic cells by macrophages¹⁵. In this study, *Drosophila* embryos containing genomic deletions were screened for a failure to efficiently engulf apoptotic cells. A deletion of the 67A-D genomic region was associated with decreased clustering of apoptotic cells within embryonic macrophages. In addition to decreased internalization of apoptotic corpses, these macrophages were strikingly smaller, yet retained normal distribution within the embryo, suggesting migratory capacity was not altered by the mutation. Further screening and mapping identified two candidate genes, *CG3428* and *CG3654*, within the deleted region. However only one, *CG3428*, was expressed in macrophages.

Transgenic expression of full-length *CG3428* specifically in macrophages rescued the engulfment defect, indicating that this was a *bona fide* engulfment gene. Sequence analysis revealed that this gene encodes an uncharacterized F-box protein and was given the name *pallbearer* (*pall*) for its role in the removal of apoptotic corpses. Interestingly, *CG3654*, also contained within the original deletion, encodes a protein with homology to the mammalian phosphatidylserine receptor (PSR), which was once thought to act as an engulfment receptor but was later found to have no obvious role in apoptotic cell engulfment^{16, 17}. It is also noteworthy that *Uch-L3*, a gene adjacent to the *pall* and the *psr*-like genes encodes a de-ubiquitylation enzyme, yet a phagocytosis-defective insertion mutation upstream of both *pall* and *Uch-L3* open reading frames did not affect *Uch-L3* expression, indicating that it was not responsible for the defective engulfment phenotype.

In addition to identifying *pall* as a novel engulfment gene, Silva *et al* examined the role of Pall in the context of the SCF-ubiquitylation apparatus¹⁵. Through biochemical analyses it was shown that Pall interacts with SkpA via its F-box domain in S2 insect cells. This was an important observation because Skp proteins tether F-box proteins to the Cul1 scaffolding protein of the SCF complex (Figure 1). Thus Pall can participate in the SCF multisubunit complex. This idea was further supported through genetic studies. The null mutants of *skpA*, *cull1* and *effete* (an SCF-associated E2 ubiquitin-conjugating enzyme) displayed defects in phagocytosis comparable to the *pall* mutant. By contrast a null mutant of Slimb, another SCF F-box protein subunit known to be involved in innate immunity, did not exhibit a deficiency in apoptotic cell engulfment, suggesting specificity of the Pall-containing SCF complex for the engulfment of apoptotic cells. In an effort to link apoptotic cell engulfment to the proteasomal pathway, dominant-negative subunits of the 26S proteasome were

expressed in transgenic flies, with embryonic macrophages from the transgenics displaying defective engulfment similar to the SCF mutants.

These results make a strong case for the requirement of a specific ubiquitylation apparatus in the efficient engulfment of apoptotic cells by macrophages. It is notable that *pall* null embryonic macrophages can still engulf apoptotic cells (~1 corpse per macrophage compared to ~3 for wild-type), indicating partial redundancy in the system¹⁵. Still these data raise intriguing and important questions not addressed by this study: What is the role of the Ub-proteasomal pathway during engulfment and what are the substrates targeted by Pall? It certainly seems that Ub-directed proteolysis is important for engulfment, but at this point there is a paucity of evidence to indicate the step at which protein degradation would be required for engulfment. The most obvious step, corpse degradation, was explored by Silva *et al* with Pall-deficient embryonic macrophages, but despite dramatically fewer numbers of corpses per macrophage compared to wild-type, the appearance of the internalized corpses was similar by electron microscopy. Currently, we do not know whether the kinetics of phagosomal maturation are affected by the *pall* mutation, which could prevent efficient uptake by an as yet unknown feedback mechanism. Although it has been shown that components of the proteasome associate with the phagosome there has been no conclusive link established between phagosome maturation and phagocytic efficiency^{9, 18}.

Another possibility is that ubiquitylation may control recognition of apoptotic cells by regulating engulfment receptors. The ubiquitylation of numerous receptors has been shown to direct their subcellular localization, degradation and signaling potential in various model systems^{19, 20}. Thus it is plausible that the SCF complex could be required for the optimal arrangement and/or transmission of signals by engulfment receptors. It remains to be seen if key engulfment receptors such as Draper and Croquemort in *Drosophila*, or their mammalian counterparts are ubiquitylated. However, it is noteworthy that Pall-deficient macrophages still possess the ability to take up AcLDL via the scavenger receptor pathway, further supporting the idea that Pall-SCF may function specifically in the context of apoptotic cell recognition by engulfment receptors¹⁵.

The two established engulfment signaling pathways known to signal to Rac and promote formation of the phagocytic cup during corpse internalization (originally based on *C. elegans* genetic studies) would provide ample targets for regulation by ubiquitylation. In mammalian cells, the components upstream of Rac include BAI1, ELMO1, Dock180, CrkII and RhoG, as well as the LRP1/MEGF10, GULP and ABCA1 proteins^{2, 3}. While ubiquitylation can regulate signaling networks, as with components of the NFκB module, precisely how ubiquitylation may control engulfment proteins is purely speculative²¹. Intriguingly, the Rac-GEF Dock180 is ubiquitylated in the membrane fractions of cultured cells²². ELMO, which binds to Dock180 and enhances Rac activation, seems to inhibit this ubiquitylation²². The extent of Dock180 ubiquitylation is unclear, as is how this ubiquitylation might affect Dock180 function during engulfment. Also, a recent report by Iioka *et al* indicates that paxillin, a CrkII-binding protein important for cytoskeletal rearrangement, is ubiquitylated and associates with the proteasome complex via a RING finger protein (XRNF185) in *Xenopus* embryos to regulate mesodermal cell motility and adhesion during gastrulation²³. Although the importance of paxillin in engulfment is poorly understood, this finding raises the possibility that CrkII may be another potential target for ubiquitylation, which could help explain the somewhat enigmatic role of CrkII in engulfment²⁴.

The work of Franc and her colleagues has brought to the fore a previously unappreciated facet of signaling during apoptotic cell engulfment. While questions regarding the role of ubiquitylation and proteasomal degradation in this process abound, this work may lead to

the identification of new players in engulfment and also presents an exciting opportunity to further understand the complexities of signaling during apoptotic cell engulfment.

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Figure 1.

Schematic representation of the proposed Pall-SCF complex. The scaffolding protein Lin19 (dCul1) links the Roc1-E2-Ub conjugated complex with SkpA-Pall and unknown engulfment-related substrate. Pall presumably promotes ubiquitylation of target proteins and subsequent proteolysis via the 26S proteasome.