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LC3-associated phagocytosis seals the fate of the second polar body in *Caenorhabditis elegans*

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1 INTRODUCTION

Cell death and clearance play key roles in disease development, progression and therapy (Poon et al. 2014). It is important to improve our understanding of these topics in order to reveal disease pathomechanisms and enable the development of novel therapeutic strategies. In our work, we establish the 2nd polar body of the nematode *Caenorhabditis elegans* as a novel genetic model system to study cell death and clearance. In this thesis, I characterize the timing of internalization of the 2nd polar body and demonstrate the involvement and function of actin regulators and trafficking proteins during internalization as well as the timely recruitment of maturation factors and their regulation. This characterization enables future research on LC3-associated phagocytosis in a well characterized model organism, which is evolutionary preserved within other species including mammals and therefore humans.

1.1 Cell death and clearance, inflammation, and disease

In a healthy human body, approximately 1 million cells die per second (Han & Ravichandran 2011). Most of them die in a programmed matter, depending on a death signal, in a process known as programmed cell death (Elmore 2007). This process is absolutely physiological and essential for the developing as well as the adult organism. During the development of the organism for example, the nervous system and the immune system depend on an overproduction of cells, which are then refined. The T-cells in the thymus which show autoimmune or no antigen reaction need to be cleared, as well as neurons which fail to form synaptic connections (Opferman & Korsmeyer 2003; Nijhawan et al. 2000). Developed tissues also depend on constant mitotic cell divisions to maintain functionality. The blood stream constantly loses white and red blood cells as well as thrombocytes which need to be replaced; the gut system constantly renews its mucosa as well as the skin its dermis. In these tissues, an equal number of cells needs to be produced and removed in order to keep cell homeostasis. Thus, cell death and cell clearance are key processes during tissue hemostasis and need to be well regulated.

Dying cells need to be cleared to avoid accumulating in tissues and to avoid inducing inflammation (Poon et al. 2014). Dying cells are cleared by migratory phagocytes (cells specialized in engulfment and degradation of other cells) as well as local cells (Poon et al. 2014). Phagocytosis of dying cells is mostly a non-inflammatory process, as there is no need for the immune system to act at sites where cells die physiologically. If apoptotic cells are not cleared, they can undergo secondary necrosis (uncontrolled cell death), leading to inflammation. Apoptotic cells are rarely detected in healthy tissues, as they are quickly cleared, therefore the presence of apoptotic cells and inflammation in a tissue has been linked to a variety of diseases (Poon et al. 2014).

A key role of the inflammatory response is to defend the organism against harmful intruders like bacteria and viruses. Inflammation leads to increased blood flow and vascular permeability to aid immune proteins in reaching the affected region. Adhesion molecules and chemokines attract leukocytes to migrate into the tissue and engulf any pathogens (Nourshargh & Alon 2014). For many years, cell death was considered to be a result of inflammation, but in fact, cell death can also trigger inflammation (Rock & Kono 2008). Infection can lead to cell death (Proskuryakov et al. 2003), but necrosis can also result from a variety of damages (hypoxia, radiation, toxins and others) where the immune response has a key role as well. During an ischemic heart attack, for example, inflammation increases blood flow to an area suffering hypoxia. Developing cancers have increased cell death rates and the immune response can lead to removal of cancerous cells neighboring the dying cells (Dunn et al. 2006). After injury, the immune response not only quickly recruits immune cells to prevent a severe infection in a new wound, but also leads to better wound healing by stimulating epithelial cells and fibroblasts to divide using the engulfed and broken-down dead cells (Rock & Kono 2008). Ergo, an inflammatory response to dying cells is valuable for the organism in case of infection or damage.

Key steps in cell death and clearance are the initiation of the cell death machinery, leading to a signaling of the dying cell for engulfment, followed by

engulfment and degradation in the phagocytosing cell. Many steps in this process can be impaired, and defects of cell death and clearance have been linked to a variety of diseases as well as disease progression (Poon et al. 2014), which are discussed below. Improving our understanding of cell death and clearance mechanisms will further reveal disease pathomechanisms and will enable the development of novel therapeutic strategies.

Cell death initiation pathways and disease. One of the best-known examples of a disease deriving from impaired cell death is cancer. Cancer can develop if cells start to proliferate unregulated or if their cell death initiation pathways are impaired (Ouyang et al. 2012). Either will result in a loss of the balance between the number of new cells deriving from mitotic divisions and the number of cells dying, leading to over-proliferation of a cell population. Certain mutations in so-called tumor suppressor genes can promote cancer development, like mutations in p53 and in some members of the bcl-2-family (Kastan et al. 1991; Benchimol 2001; Llambi & D. R. Green 2011). These genes are known to promote cell death, especially after irreparable damage to DNA has occurred. Loss of their function means losing the ability to initiate cell death pathways after damage which can result in the development of cancer. p53 mutations can be found in over half of all cancers, therefore targeting it and other tumor suppressor genes could be a novel approach in cancer therapy, but further research is needed to bring these methods to the stage of clinical applicability (A. J. Levine 2019; Campbell & Tait 2018). Thus, investigating cell death initiation pathways has improved our understanding of cancer development and further research will likely open novel individual therapeutic strategies for cancer patients.

Corpse recognition and disease. After a cell has initiated its cell death machinery, it must signal for engulfment. Exposing the lipid phosphatidylserine (PS) on the outer leaflet of the cell's bilayer membrane is one of the best known "eat-me" signals (Bratton et al. 1997; Fadok et al. 2001). **M**ilk **f**at **g**lobule-**E**GF factor **8** protein (MFGE8) binds to PS and promotes cell engulfment (Hanayama et al. 2002). Impaired cell corpse recognition has been shown to influence disease progression. Atherosclerosis for example is a leading cause of death in

western societies. One cause for the disease is chronic inflammation in the vascular wall leading to the formation of plaques. Impaired phagocytosis of macrophages and accumulation of apoptotic cells that undergo secondary necrosis has been reported in atherosclerotic plaques. This leads to plaque expansion and potential rupture leading to acute ischemia of the downstream tissue (Schrijvers et al. 2005). A lack of engulfment factors like MFGE8 has been shown to accelerate atherosclerosis (Tabas 2009). Another disease that has been linked to MFGE8 defects is inflammatory colitis. In mice models with induced colitis, MFGE8 mutants showed more severe colitis than healthy mice (Kusunoki et al. 2012). Similar roles of MFGE8 have been identified in inflammatory diseases, systemic lupus erythematosus-type autoimmune disease, age-related diseases and tumors (B.-Z. Li et al. 2013). Ergo, defects in recognizing dying cells can severely influence disease progression.

Corpse engulfment and disease. Impaired phagocytosis has been linked to several types of inflammatory lung disease, including chronic obstructive pulmonary disease (COPD), cystic fibrosis and pulmonary fibrosis (Hodge et al. 2003; Morimoto et al. 2012; MD et al. 2002). Adult patients suffering from severe asthma and children suffering from poorly-controlled asthma have also been shown to have alveolar macrophages with defective clearance function (Huynh et al. 2005). These findings may further explain the effectiveness of steroid treatment as it enhances phagocytosis by macrophages (Liu et al. 1999). Thus, impaired cell corpse engulfment, just like defects in cell corpse recognition pathways, significantly influence the course of a disease and poses an attractive target for therapies.

Phagocytosis and autoimmune disease. Phagocytosis of apoptotic cells also plays a key role in immune self-tolerance. Uncleared apoptotic cells eventually undergo secondary necrosis and burst, dispersing their cellular contents (Elmore 2007). These cellular contents are normally compartmentalized within the cell and their extracellular presence increases the risk of being recognized as non-self by the immune system, leading to production of auto-antibodies and autoimmune disease (Muñoz et al. 2010; Poon et al. 2014). Additionally, the

phagocytosis of apoptotic cells by dendritic cells results in cross presentation of the phagocytosed peptides on MHC class I molecules. CD8+ T-cells bind to MHC I and can react when foreign antigens (i.e. from a viral infection or after cancerous mutations) are identified. But presenting the auto-antigens on MHC class I molecules is also essential in keeping the immune system trained to ignore them and therefore promote self-tolerance (Belz et al. 2002). Impaired phagocytosis can therefore lead to autoimmunity. The autoimmune disease systemic lupus erythematosus (SLE) has been linked with a variety of engulfment defects, including MFGE8 polymorphisms (Hu et al. 2009; Yamaguchi et al. 2010). Also defects of the complement system, especially C1q polymorphisms, which promote the engulfment of apoptotic cells, are highly associated with SLE (Botto & Walport 2002). Interestingly, not all cell clearance defects result in autoimmune disease. Loss of CD14 or mannose binding lectin (MBL) result in apoptotic cell clearance defects, but not autoimmune disease or inflammation (Devitt et al. 2004; Stuart et al. 2005). Further research is needed to find possible anti-inflammatory regulators that act in these cases, which could open novel therapeutic strategies to prevent autoimmune disease (Poon et al. 2014).

Bacterial clearance and disease. Removing pathogens like bacteria is a core function of our immune system. After recognition of pathogen-associated molecular patterns (PAMPs), innate immune cells like neutrophils are often the first cells to react and engulf pathogens (Serhan et al. 2007). These cells then undergo apoptosis and are mostly cleared by macrophages and local epithelial cells (Savill et al. 1989; Haslett 1999). Clearing the cell that has engulfed the pathogen seems to be beneficial as two rounds of pathogen clearance could prevent pathogen survival in neutrophils. Indeed, pharmacological elevation of neutrophil apoptosis in pneumococcal meningitis reduces brain hemorrhage and accelerates recovery (Koedel et al. 2009). However, some pathogens, like *Chlamydia pneumoniae* have evolved a 'Trojan horse' strategy, leading to infection of the macrophage that cleared the infected neutrophil (Rupp et al. 2009). Thus, effective phagocytosis of dying cells can help clear infections, but sometimes also spreads disease.

Negative consequences of effective cell clearance in cancer. Efficient cell clearance can also drive diseases, such as cancer. In tumors, high levels of cell death and clearance occur, and non-inflammatory clearance can lead to tumor-specific immunity, as apoptotic cell death releases anti-inflammatory mediators and the tumor antigens are constantly presented on MHC class I molecules (Gregory & Pound 2010; Belz et al. 2002). Manipulating cell clearance to become more inflammatory could therefore be therapeutically beneficial in generating an antitumor immune response (Gregory & Pound 2010). However, turning non-inflammatory into inflammatory cell death bears serious risk, as this could lead to systemic inflammation, dispersion of DAMPs, and promote autoimmune disease development. Furthermore, inflammation itself promotes cancer development and could therefore be counterproductive (Herrmann et al. 1998; Coussens & Werb 2002; Poon et al. 2014). One approach to this is the application of Annexin V, which interferes with the recognition of dying cells by masking the externalized PS, thereby promoting an inflammatory response against cancer cells (Bondanza et al. 2004). Some chemotherapeutics like anthracyclines have also been shown to promote immunogenic cell death by augmenting the antitumor immunity dependent on caspases, dendritic cells and CD8+ T-cells (Casares et al. 2005; Obeid et al. 2006; Kroemer et al. 2013). Thus, apoptotic cell death promotes immunogenic cancer tolerance, but promoting inflammatory cell death can determine the long-term success of cancer therapies by promoting an immune response against unwanted cells (Kroemer et al. 2013).

Promoting immune tolerance using apoptosis. Using the anti-inflammatory effects of apoptosis to promote immune tolerance also promises new therapeutic strategies for transplant patients. In most cases, transplant patients must take immunosuppressive drugs to prevent allograft rejection, bearing serious side-effects, including a higher rate and more severe course of infections, higher risk of malignancy, bone marrow suppression and cytopenia and higher risk of cardiovascular diseases (D. C. Hsu & Prescr 2009). Experimental research is investigating whether inoculation of apoptotic cells from the donor could promote immune tolerance in transplant recipients. Indeed, various mice and cell

culture models showed improved transplant engraftment and survival after injection of donor cells that have been induced to undergo apoptosis. Furthermore, a recent clinical trial demonstrated safety, tolerability and potential efficacy of this strategy in humans (Dangi et al. 2018). Ergo, apoptotic anti-inflammatory cell death could develop to be an attractive strategy in transplant medicine.

In summary, effective cell death and clearance is critical in human health; defects in cell death and clearance have been linked to many disease pathologies. Research in humans and model organisms has led to and will continue to lead to a more profound understanding of the molecular pathways and its components, which is essential for developing novel therapeutic strategies. Especially key will be the identification of factors deciding whether cell death is immunogenic or anti-inflammatory. Opening the possibility to interfere in this process bears attractive potential for novel therapy schemes in autoimmune and inflammatory diseases.

1.2 Cell death

Most physiological cell deaths are programmed cell deaths. Programmed cell death involves the energy-dependent activation of molecular pathways that lead to the controlled death and clearance of the cell, thereby preventing an inflammatory reaction. However, after damage, cells can also die in an uncontrolled manner, known as necrosis, which is followed by an inflammatory response. Dead cells need to be cleared (see section 1.3), as cells that die via apoptosis can eventually become necrotic, known as secondary necrosis (Leist et al. 1997).

1.2.1 Programmed cell death

Programmed cell death (PCD) can be induced by the cell itself, for example after damage beyond repair (e.g., by radiation, toxins or hypoxia), or by the absence of external stimuli, such as hormones that constantly suppress the initiation of cell death pathways. Cell death can also be induced by external stimuli,

such as a death ligand that binds to a death receptor of the cell, thereby triggering a molecular cascade that leads to cell death (Elmore 2007). Another external stimulus that will kill a cell is the recognition of an antigen-bearing cell by a cytotoxic T-cell. The T-cell can either use the death ligand/receptor FasL/R (Fatty acid synthetase) (Brunner et al. 2003) or the membrane-perforating protein Perforin to induce cell death pathways (Trapani & Smyth 2002). These pathways lead to PCD by an intracellular signaling pathway known as apoptosis.

Although apoptosis can be triggered by a variety of events and effector pathways, they all lead to a common execution pathway in the end. This includes activation of the cysteine-aspartic proteases (caspase) -3, -6 and -7, known as the “effector caspases”. These caspases cleave various substrates including cytoskeletal, plasma membrane and nuclear components, thereby leading to the morphological changes of apoptosis, which are even visible using light microscopy (Slee et al. 2001). Caspase-3 plays a key role in the execution pathway, as it is activated by any of the cell death initiation pathways. Caspase-3 activates a variety of other factors promoting the cell’s degradation, including the endonuclease CAD (Sakahira et al. 1998) and Gelsolin which cleaves actin, leading to disorganization of the cytoskeleton, intracellular transport and cell division (Kothakota et al. 1997). Another factor activated by caspases is the transmembrane protein Xkr8 (Segawa et al. 2014). Xkr8 activity leads to PS externalization on the dying cell (Suzuki et al. 2016), presenting an “eat-me” signal to surrounding cells. This results in clearance of the cell, preventing the dispersion of cellular components and promoting the release of anti-inflammatory signals (Kurosaka et al. 2003). Thus, activation of caspases initiates cellular degradation as well as signaling for clearance by phagocytosis.

It is worth noting that there are more forms of PCD than apoptosis. All of these pathways are characterized by energy-dependent activation of genes and molecular cascades that lead to cell death and clearance. The term apoptosis was originally introduced by (Kerr et al. 1972) to describe the microscopic phenotype of dying cells, including cell shrinking, condensation of chromatin due to DNA-fragmentation and membrane blebbing (apoptotic bodies) and only later

included the molecular characteristics such as caspase activation. Today, other microscopic and molecular phenotypes that are distinct from apoptosis have been described (Elmore 2007). There are forms of cell death that have features of necrosis, like cell swelling and disruption of the cell membrane, but still require the energy-dependent activation of genes and molecular cascades and are therefore classified as programmed necrosis (Proskuryakov et al. 2003). Forms of programmed necrosis involving distinct molecular pathways include necroptosis, ferroptosis, and pyroptosis (Dong et al. 2015).

Another type of cell death is autophagic cell death (B. Levine 2005; Galluzzi et al. 2008). Autophagy is a catabolic process involving formation of double-membrane structures around cell organelles and macromolecules, resulting in their intracellular degradation (Ohsumi 2001; C.-W. Wang & Klionsky 2003). It plays a crucial role in cell survival during times of starvation, but may also induce cell death when a certain stress limit is exceeded (L. M. Schwartz et al. 1993; Gozuacik & Kimchi 2004). This type of cell death is distinct from apoptosis and programmed necrosis as it has its own effector pathways, including activation of PI3 kinase type III, beclin-1 and two ubiquitin-like conjugation systems leading to the formation of autophagosomes (Tsujimoto & Shimizu 2005). However, it is not always distinguishable from other types of cell death, as autophagy activity within the dying cell could act as a rescue mechanism to promote cell survival.

The above-mentioned pathways can also happen simultaneously in a single cell, trigger or inhibit one another (Nikoletopoulou et al. 2013). Whether a cell dies by apoptosis, other forms of PCD or necrosis can depend on the specific death signal, the tissue and its developmental stage (Fiers et al. 1999; Zeiss 2003). Although, apoptosis is a mostly immunologically silent process, it can also induce inflammation e.g. when apoptosis is induced by a viral or bacterial infection of the cell. Thus, the cause of death determines whether or not an inflammatory response is needed (Elmore 2007).

In summary, there are three main types of PCD: apoptosis, autophagy and programmed necrosis, all with various subtypes that use different pathways to kill the cell. The various types of PCD are connected and the way a cell dies

depends on various factors and can change during the dying process. The components of the apoptotic pathway are relatively well characterized, but factors in other PCD pathways and the connections between pathways are still left to be identified. A better understanding of cell death mechanisms is important, especially in light of the accumulating evidence of its influence on disease origin and progression (see section 1.1).

1.2.2 Necrosis

When a cell has experienced severe damage to an extent that energy-dependent programmed cell death is not an option, it becomes necrotic. Necrosis involves swelling of the cell, karyolysis and disruption of the cell's membrane resulting in release of cytoplasm and organelles into the extracellular matrix. These released molecules are referred to as damage-associated molecular patterns (DAMPs). DAMP release causes inflammation in the tissue by being inflammatory in itself and by recruiting inflammatory cells. For example, the nuclear High Mobility Group Box 1 protein HMGB1, uric acid or even DNA have inflammatory characteristics. DAMP release can also mediate inflammation through other factors, like cleaving extracellular matrix molecules, which are then recognized by receptors (Rock & Kono 2008). Programmed necrosis as a type of programmed cell death also results in DAMP release and therefore triggers inflammation (Pasparakis & Vandenabeele 2015). Thus, losing the integrity of the cell's membrane plays a key role in promoting an inflammatory response to cell death.

Typically, apoptosis is an anti-inflammatory process, but an apoptotic cell can become inflammatory if it undergoes secondary necrosis. Secondary necrosis occurs after a cell has initiated its cell death pathways but is not cleared. The initiation and execution of cell death depends on energy. When ATP availability becomes deficient, the cell starts to die in an uncontrolled manner, eventually becoming necrotic (Leist et al. 1997). This process has the same consequences as primary necrosis, including cell swelling and disruption of the cell membrane, leading to DAMP release and inflammation. Thus, cell clearance is a key step during programmed cell death to prevent an inflammatory response.

1.3 Cell corpse clearance

Phagocytosis is the process of engulfing relatively large particles like bacteria or cell corpses. Dying cells exposing phosphatidylserine can be recognized by PS receptors on the engulfing cell and signal to induce phagocytosis (Bratton et al. 1997; Fadok et al. 2001). This signaling leads to actin rearrangements, reforming the plasma membrane of the engulfing cell into a phagocytic cup that closes around the corpse. The phagocytosed material ends up in a vesicle called a phagosome (Ravichandran & Lorenz 2007). An engulfed cell corpse will therefore end up in a vesicle with two bilayer membranes: one membrane derived from the engulfing plasma membrane, the other from the plasma membrane of the phagocytosed cell corpse. The content of the phagosome then needs to be degraded and recycled.

After internalization, the phagosome will mature by gradually acidifying and fusing with endolysosomes that contain the digestive hydrolases necessary for degradation of the phagosome contents (Kinchen & Ravichandran 2008). The maturation process is characterized by the stereotyped presence of maturation factors, which mark important steps in the process. After internalization, the nascent phagosome becomes an early endosome as indicated by the presence of the small GTPase Rab5. Rab proteins are GTPases that switch between membrane bound and cytosolic states, recruit different proteins to endosomes, and catalyze organelle-organelle as well as organelle-membrane fusion (S. L. Schwartz et al. 2007). As the phagosome matures to become late endosome-like, Rab5 is exchanged for Rab7. LAMP1 (**l**ysosome **a**ssociated **m**embrane **p**rotein 1) marks the fusion of late endosomes and phagosomes with lysosomes (Fairn & Grinstein 2012). The phagosome is considered a phagolysosome once it achieves low pH-levels (Kinchen & Ravichandran 2008).

In the end, the phagocytosed cargo is digested into its nucleotides, amino acids, fat and sterols, which are transported out of the phagolysosomes to be reused. Glucose, amino acids, and nucleotides get transported via specific transporters into the cytoplasm, some of which are not yet identified (Mony et

al. 2016; Fujiwara et al. 2016). There are many known defects in lysosomal processing of these materials leading to so-called lysosomal storage disease, often presenting with central nervous system dysfunction and debilitating symptoms including cognitive and motor decline (Schultz et al. 2011). Research on lysosomal degradation has led to a much better understanding of these diseases and to the development of promising therapies (Parenti et al. 2015), but a lot is yet to be uncovered and needs to be researched to improve our understanding especially of phagolysosomal degradation and disease pathomechanisms.

Metazoa (multicellular animals) have developed a variety of cells that are specialized for phagocytosis. Depending on the tissue and type of cell death, macrophages, dendritic cells and neutrophils will engulf and degrade cell corpses (Gregory 2009). However, non-professional phagocytes like epithelial cells and fibroblasts also engulf dying cells. While all tissues contain cells undergoing apoptotic cell death, some deal with high numbers of cell corpses, including the bone marrow with its high cell turnover during hematopoiesis, the spleen as part of the mononuclear phagocyte system recycling red blood cells, and the thymus in which T-cells develop and 80% undergo apoptosis when they are autoimmune reactive (Hochreiter-Hufford & Ravichandran 2013; Poon et al. 2014). Fragments of degraded peptides of the body's own cells are then presented by the major histocompatibility complex (MHC) class I molecules of antigen-presenting cells (APCs, mostly dendritic cells) to establish and maintain immune self-tolerance (Bellone et al. 1997; Albert et al. 1998; F. P. Huang et al. 2000; Belz et al. 2002). As phagosome maturation and degradation can significantly influence a cell's phagocytic capacity (Wu et al. 2000; Krieser et al. 2002; Schrijvers et al. 2005; Park et al. 2011), some cells have superior phagocytic capacity, but since dead cells need to be cleared in all tissues, there are cells with phagocytosing activity in every tissue.

1.3.1 LC3-associated phagocytosis

Certain phagocytic cargo, including cell corpses, has been shown to be cleared by LC3-associated phagocytosis (LAP) by both professional, as well as non-professional phagocytes (X. Wang & C. Yang 2016). LAP is a process in

which factors from classical phagocytosis (see section 1.3) as well as factors from macroautophagy (hereafter autophagy) are involved. For autophagy, an isolation membrane (phagophore) extends around intracellular cytosolic components and wraps them in a double membrane autophagosome for degradation. Phagocytosis on the other hand engulfs extracellular targets and degrades them. Phagocytosis of cells, cell parts or bacteria will also lead to double-membrane phagosomes, with one membrane deriving from the engulfing cell's plasma membrane, the other from the membrane of the internalized cargo (see Fig. 1B). For LAP, the internalized phagosome matures similar to classical phagocytosis, including recruitment of maturation factors like Rab5 and Rab7 before fusing with lysosomes and becoming a LAMP1-positive phagolysosome; but additional factors known from the autophagy machinery are also recruited and necessary for phagosome-lysosome fusion and degradation (Sanjuan et al. 2007; Florey et al. 2011; Martinez et al. 2011; Kim et al. 2013; Fazeli et al. 2016). This includes proteins from the Atg8 family, such as LC3 and GABARAP, which have to be lipidated by Atg7 for their activity in LAP and autophagy (Tian et al. 2010).

Sometimes it is hard to distinguish between LAP and autophagy, as both present with LC3-positive vesicles, but there are mechanistic differences. First, engulfment receptors are not needed for autophagy, as the target is located inside the cell. Similarly, certain factors from autophagy, especially from the autophagy pre-initiation complex that initiates the formation of the double-membrane phagophore, are dispensable for LAP, as the cargo is only wrapped in the phagosome membrane after engulfment. Additionally, the PI3K-complex differs in some components, e.g. Atg14 (EPG-8) is exclusively found in the autophagy complex, whereas the RUN domain Rubicon is only found in LAP (P. Yang & Zhang 2011; Martinez et al. 2015; Fazeli & Wehman 2017b).

The functional role of LAP has been debated, but evidence suggests that its role may be to clear membrane-wrapped debris. For example, LAP has been shown to clear bacteria, fungi, cell corpses, shed photoreceptor outer segments, and midbody remnants released after cytokinesis (Sanjuan et al. 2007; J. Huang et al. 2009; Sprenkeler et al. 2016; Florey et al. 2011; Martinez et al. 2011; Fazeli

et al. 2016; Fazeli & Wehman 2017b; Kim et al. 2013). Defects in LAP have been linked to some diseases, e.g. factors involved in autophagy and LAP have been shown to protect against age-related macular degeneration (Mitter et al. 2014). Furthermore, mice lacking LC3-associated phagocytosis factors produce auto-antibodies and develop a SLE-like autoimmune disease (Martinez et al. 2016). Therefore, investigating LAP could help to understand the mechanisms behind age-related and autoimmune disease.

Despite these important observations, there are many open questions about LAP, including the role of LC3 during phagolysosomal degradation. Intriguingly, LC3 has been shown to be needed for inner membrane degradation of autophagosomes (Tsuboyama et al. 2016). After internalization, the phagosome consists of at least two membranes, just like the double-membrane autophagosomes. Therefore, it should be tested whether LC3 plays a role in the degradation of internalized cargo membranes or whether it has other functions in phagosome maturation and cargo degradation.

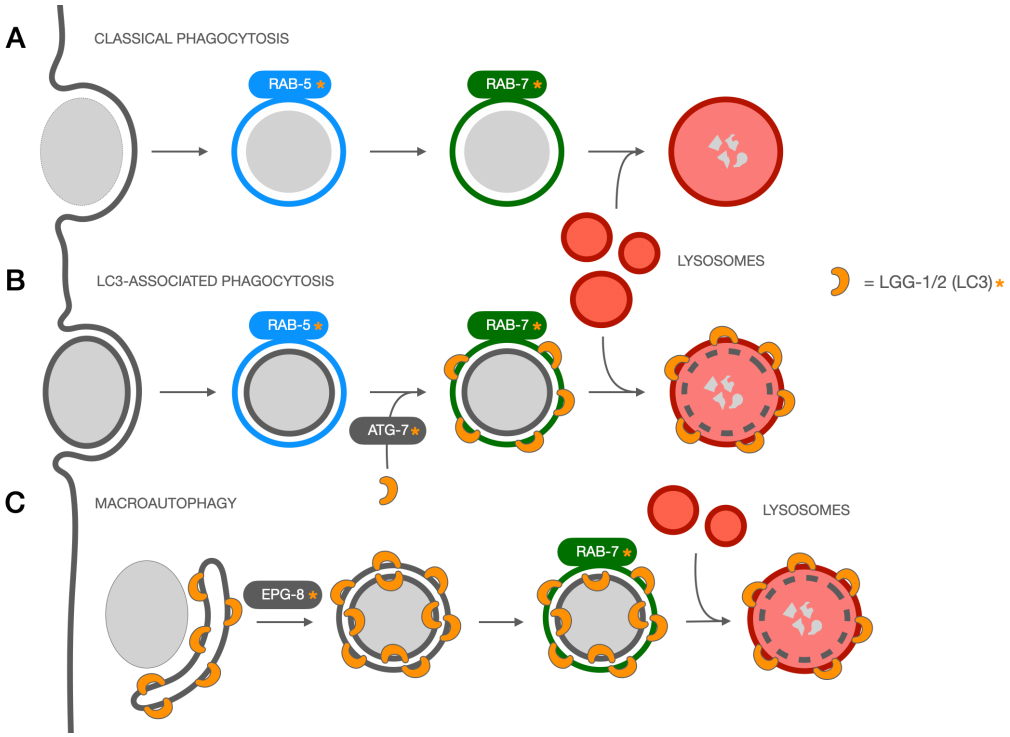


Figure 1 – Key differences between classical phagocytosis, LC3-associated phagocytosis (LAP) and macroautophagy.
(A) Classical phagocytosis engulfs the cargo, the phagosome matures by acquiring the Rab GTPases RAB-5 and RAB-7, leading to fusion with lysosomes and ultimate degradation.
(B) For LAP, the LC3 homologs LGG-1 and LGG-2 are recruited to the phagosome in an ATG-7-dependent manner. The role of LC3 recruitment during LAP is unclear.
(C) Macroautophagy involves the formation of a double-membrane phagophore around the target. Some factors, including EPG-8, are exclusively found in macroautophagy and not LAP. LC3 promotes inner membrane degradation during macroautophagy.
Orange stars indicate C. elegans proteins investigated in this work.

1.4 Polar bodies

To gain a better understanding of the mechanisms of cell death and cell corpse clearance, we wanted to study the death and clearance of a conserved cell that dies in a stereotyped fashion. For these reasons, we studied polar bodies that derive from female meiosis and die after their extrusion.

In order to produce progeny, 99.9% of eukaryotes at least occasionally sexually reproduce (Otto 2008). Sexual reproduction includes oocyte fusion with a sperm to form a zygote, the first cell of a newly developing organism. With the fusion of these two cells, their genome also gets combined. As polyploidy (a cell containing more than two homologous chromosomes) is lethal in most cases

(Plachot 2001), these fusing cells have to be haploid, thus consisting of half of the normal set of chromosomes. To form haploid cells, spermatocytes and oocytes undergo a process called meiosis. Female meiosis starts with a germ line cell, the primary oocyte, which undergoes two asymmetric cell divisions, discharging three quarters of its DNA into tiny cells called polar bodies. In the first meiotic cell division (Meiosis I) homologous chromosomes get segregated, half of them ending up in the diploid 1st polar body. In the second meiotic cell division (Meiosis II), sister chromosomes get segregated to generate a haploid oocyte. The discharged chromosomes end up in the haploid 2nd polar body. In male meiosis, a primary spermatocyte gives rise to four spermatids, thus no polar bodies are formed in this process.

Polar bodies are formed by an extreme type of asymmetric cell division referred to as extrusion. One pole of the meiotic spindle is attached to the cell cortex in a perpendicular orientation, which aids the extrusion of the polar body during the meiotic cell division (Fabritius et al. 2011). Extrusion leads to an extremely uneven distribution of cytoplasm between the polar bodies and the oocyte, which can be 100-fold larger in volume (Gitlin et al. 2003).

The asymmetry of the meiotic cell division has been shown to be essential for polar body death. Recent research with mice embryos has shown that polar bodies formed by symmetric cell division with an even distribution of cytoplasm can be fertilized, leading to two zygotes that compete for space and later fuse together, resulting in a viable chimeric mouse (Otsuki et al. 2012). Thus, the uneven distribution of cytoplasm seems to significantly contribute to polar body death. Even human polar bodies could be fertilized (Bieber et al. 1981). Fertilizing the 2nd polar body as well as the ovum could theoretically result in twins or chimeras, although there is no confirmed report of such a person so far (Machin 2009). Thus, the uneven distribution of the cytoplasm between the oocyte and the polar bodies likely contributes to polar body death while promoting the health of the oocyte and resulting embryo.

Although polar bodies are very small, they are still considered a cell; they contain a nucleus, endoplasmic reticulum, Golgi, and mitochondria (Zamboni

1970; Dalton & Carroll 2013). In some organisms, the 1st polar body undergoes a second division (Schmerler & Wessel 2011), and in some species, polar bodies continue to divide and can even form whole organs. For example, in some scale insects (family: *Diaspididae*), polar bodies fuse together, creating a nutritive tissue, the bacteriome. Polar bodies can also form a protective tissue around the embryo of the parasitic wasp *Copidosoma floridanum* (Schmerler & Wessel 2011). Thus, dying polar bodies can be considered to be the first cells to die during embryonic development in many species.

Although polar bodies die in many animals, the mechanism of their death is not understood. In bovine embryos, polar bodies undergo programmed cell death as they present with fragmented DNA (Matwee et al. 2000). However, polar bodies in mice still die after exposing the embryo to caspase inhibitors (Zakeri et al. 2005), suggesting that polar body death is a caspase-independent form of programmed cell death, not apoptosis. Mice polar bodies have also been shown to externalize the "eat-me" signal phosphatidylserine, which is often taken as a sign of apoptotic cell death, but they can also be stained with membrane-impermeant dyes like propidium iodide (PI), suggesting that they become necrotic (Fabian et al. 2012). In conclusion, mammalian polar bodies are likely to undergo a form of non-apoptotic programmed cell death.

As polar bodies die in humans, they are thought to provide no further function during human embryogenesis. Indeed, human polar bodies are routinely removed and used for genetic testing for inheritable diseases of the mother, like cystic fibrosis (Verlinsky et al. 1997). However, there is discussion whether laser drilling the zona pellucida and removing the polar body for preimplantation genetic diagnosis could affect embryonic development and the health of these humans (I. Levin et al. 2012; Hammoud et al. 2010). A study looking at health and development of humans on which preimplantation genetic diagnosis were performed did not find any significant impact (Desmyttere et al. 2009), but there is still a need for a randomized controlled trial, as has been recommended by the Preimplantation Genetic Diagnosis International Society. Thus, it is important to

investigate the death and clearance of polar bodies in order to improve our understanding of cell death and clearance in early embryos and its implications for the development of the organism.

1.5 *C. elegans* as a model organism

To study cell death and clearance of polar bodies we needed a model organism that has been well characterized, produces a high number of progeny in a short period of time with low cost, and limits the ethical conflicts of investigating embryos*. Caenorhabditis elegans* worms appeared ideal as they matched all criteria.

C. elegans was first introduced as a genetic model to research cell death and clearance by Sydney Brenner in 1963 and has since been intensively studied (Corsi et al. 2015). During embryonic development, 131 cells undergo programmed cell death of the 1,090 cells born (Horvitz 1999). The time and position of each's cell death is known and consistent (Sulston 1976; Sulston et al. 1983) and can be observed using differential interference contrast (DIC) microscopy (Robertson & Thomson 1982). Furthermore, cell death and cell corpse clearance are not essential for the viability of *C. elegans* worms. Thus, disrupting genes required for cell death does not prevent worms from producing progeny, allowing studies on worm lines with impaired cell death and its effects (H. M. Ellis & Horvitz 1986). The work on the *C. elegans* cell lineage and cell death was rewarded with the Nobel Prize for Medicine in 2002 to Sydney Brenner for introducing *C. elegans* as a model organism, to John E. Sulston who mapped the cell lineage and showed that specific cells undergo programmed cell death during embryogenesis, and to H. Robert Horvitz for identifying key genes regulating cell death, their interaction and that they have human homologues (Brenner 2003; Horvitz 2003; Sulston 2003). Thus, C. elegans is an established model for researching the conserved mechanisms of cell death and corpse clearance.

Humans share a lot of similarities in molecular and cellular processes with worms. 38% of *C. elegans* genes have predicted human orthologs (Shaye & Greenwald 2011), while 60% of human genes have *C. elegans* orthologs. Furthermore, 40% of human genes known to be involved in disease have *C. elegans*

orthologs. *C. elegans* also has a more compact genome than humans with fewer duplicated genes (Hodgkin 2001), allowing insight into molecular function from single gene studies. Many diseases have been studied using *C. elegans,* including diabetes and obesity, cancer, Alzheimer's, Parkinson's and Huntington's disease, muscular dystrophy, ion channelopathies, innate immunity, depression, pain and aging (Kaletta & Hengartner 2006). Additionally, researching basic biological principles and pathways has led to a much more profound understanding of the human body and enabled further research and development of therapies (Kaletta & Hengartner 2006; Corsi et al. 2015).

C. elegans is a small nematode, with adults measuring around 1 mm in length. Since the worms are transparent, its cells are microscopically observable throughout embryogenesis, larval, and adult stages (see Fig. 2A-B). By tagging fluorescent proteins to specific proteins or cellular compartments, resolution can be further enhanced, allowing developmental processes and protein-protein interactions to be visualized *in vivo* (Chalfie et al. 1994; Feinberg et al. 200; see Fig. 2C). The ability to tag specific targets in an animal with fluorescent proteins was first demonstrated in *C. elegans*. This resulted in the Nobel Prize for Chemistry in 2008 being awarded to Osamu Shimomura for isolating GFP from the jellyfish *Aequorea victoria*, to Martin Chalfie for showing the possibility to tag biological phenomena in *C. elegans,* and to Roger Y. Tsien who revealed why GFP fluoresces and introduced further colors, which enabled multicolor tagging of different targets in one cell (Shimomura 2009; Chalfie 2009; Tsien 2010).

Other practical advantages of *C. elegans* as a model organism in research include their short life cycle, abundant progeny, and simplicity (Corsi et al. 2015). *C. elegans*' life cycle from egg to egg-laying adult is rapid, taking around 3 days; embryogenesis lasts around 16 hours. The worms are self-fertilizing hermaphrodites producing hundreds of progeny over the course of a few days. Males are rarely generated (around 0.2% of progeny), but male progeny can be increased by heat shock. Males are useful to cross worm strains, which otherwise "are driven to homozygosity" (Brenner 1974). The worms are cultured on agar plates with *E. coli* as food. When they run out of food, *C. elegans* worms can enter a

dauer stage, surviving for months without being fed. Worm populations can also be frozen and revived after years as needed. Another advantage of studying *C. elegans* is the ability to create transgenic or genetically-modified worms. Furthermore, gene function can be rapidly tested using RNA interference. Target protein levels can be knocked down easily by feeding the worms bacteria generating dsRNA (Timmons & Fire 1998; Fire et al. 1998). Andrew Z. Fire and Craig C. Mello were rewarded the Nobel Prize for Medicine in 2006 for their discovery of the effects of injecting dsRNA into *C. elegans* (Fire 2007; Mello 2007).

Thus, *C. elegans* is an attractive genetic model system to study conserved molecular processes, cell biology, and to model human disease.

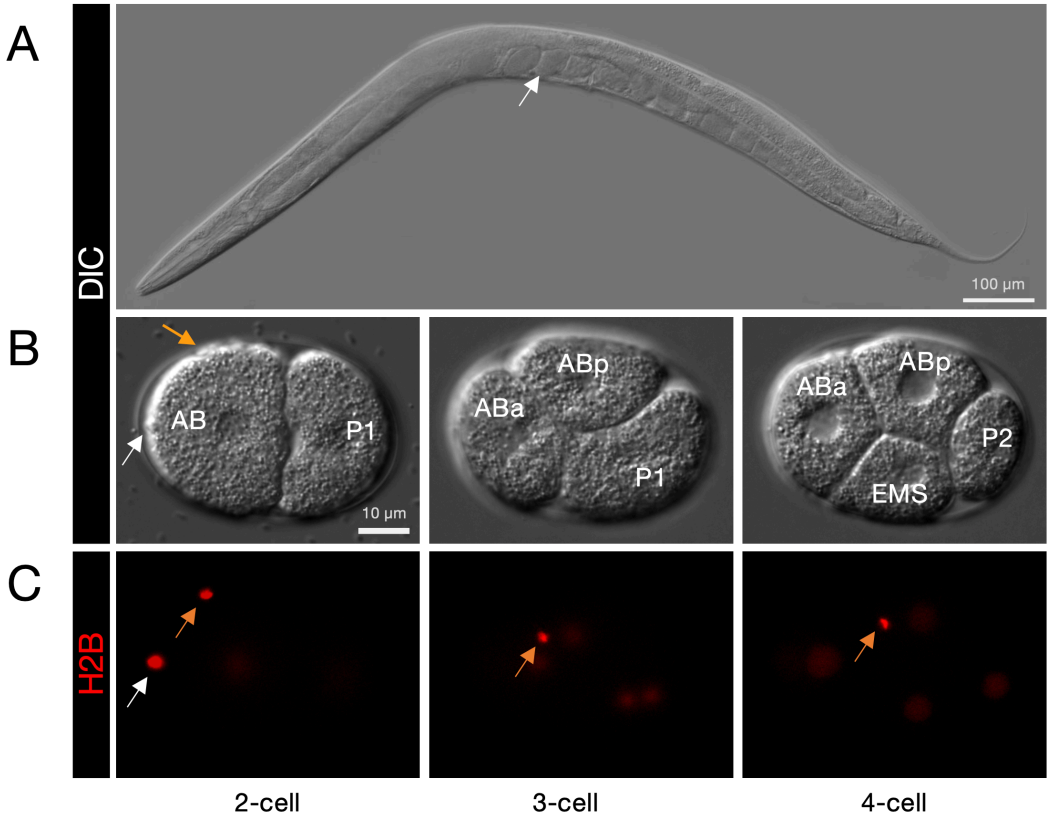


Figure 2 – *C. elegans*, the adult worm and a young embryo's first cell divisions.
(A) The adult worm measures around 1 mm in length. The white arrow indicates an exemplary embryo in the gravid hermaphrodite, others can be found further anterior and posterior. *This picture "Adult Caenorhabditis elegans" is shared by Zaynep F. Altun at wormatlas.org to be used under the Creative Commons BY-SA 2.5 licens. Arrow and scale bar were added.*
(B) DIC images of a *C. elegans* embryo during the 2-, 3- and 4-cell stages. White arrow indicates 1st polar body, orange arrow indicates 2nd polar body. After the polar body has moved in between the ABa- and ABp-cell it is no longer trackable by DIC microscopy. *Cell names are labeled. AB=anterior blastomere, P=germ line cell, EMS=endomesodermal precursor cell.*
(C) Fluorescent pictures of the same embryo expressing an mCherry::H2B histone fluorescent marker. The marker enables easy tracking of the polar body even after engulfment, which has already occurred in the 4-cell stage embryo shown here. The 1st polar body is not displayed in the 3- and 4-cell stage images as it is positioned in another optical section as the 2nd polar body.

1.5.1 Programmed cell death and corpse clearance in *C. elegans*

Most of our understanding of programmed cell death derives from studies with *Caenorhabditis elegans,* especially since the mechanisms of programmed cell death in *C. elegans* are conserved in humans (Adams 2003; Horvitz 2003; Danial & Korsmeyer 2004; Fuchs & Steller 2011; Fond & Ravichandran 2016). In the invariant *C. elegans* cell lineage (Horvitz 1999), it is predetermined which 131 cells die, when and where (Sulston 1976; Sulston et al. 1983), which makes *C. elegans* an excellent model organism to study programmed cell death.

Cells that die via apoptosis first undergo a “specification” phase, which selects the cell to die. The cell death machinery then needs to be activated in this cell (“activation phase”). In the end, the cell death machinery executes the cell death in the “execution phase,” leading to the ultimate death of the cell and signaling for engulfment by neighboring cells (Horvitz 1999; Conradt et al. 2016).

Two of the key genes acting as cell fate switches during embryonic development are *ced-3* and *ced-9* (**ce**ll **d**eath abnormality genes). CED-3 is the *C. elegans* homolog of the vertebrate caspase-3 that plays a central role in apoptosis (Rueda et al. 1999). CED-3 acts as a death-promoting factor that induces mitochondria elimination, the suppression of cell-survival signals and nuclear degradation (R. E. Ellis & Horvitz 1991). CED-3 can be inhibited by CED-9 activity (Fig. 3), which is essential for embryonic development. Cells that would normally live undergo programmed cell death in *ced-9* mutants (Hengartner et al. 1992). CED-9 is similar to the human proto-oncogene **B**-**c**ell **l**ymphoma 2 or bcl-2 (Hengartner & Horvitz 1994), which also has an anti-apoptotic effect in mammals (Adams & Cory 2001). Interestingly, similar to polar bodies, cells that are programmed to die in *C. elegans* are mostly formed by asymmetric cell division, resulting in the smaller cell dying. Consequently their fate has been decided before their birth (Sulston et al. 1983). Indeed, several genes promoting cell polarization and asymmetric cell division have been identified to significantly contribute to cell fate and cell death (Conradt et al. 2016).

When pro-apoptotic factors outweigh anti-apoptotic factors, the cell death machinery gets activated, mostly by activating CED-3. Activated CED-3 (acCED-3) activates a variety of nucleases that catalyze DNA fragmentation, which can be visualized by TdT-mediated dUTP nick end labeling (TUNEL) (Wu et al. 2000). CED-3 also activates the **w**orm **a**poptosis-**i**nducing factor homolog WAH-1, which acts in a complex with other factors as a nuclease (X. Wang et al. 2002). WAH-1 also plays a key role in externalizing phosphatidylserine (PS), which acts as an “eat-me” signal for apoptotic cells to be phagocytosed (Fig. 3). WAH-1 accomplishes this by binding to the membrane-localized **scra**m**b**lase SCRM-1, whose activation leads to PS exposure on the outer leaflet of the

plasma membrane (X. Wang et al. 2007). Another factor leading to PS externalization after cleavage by acCED-3 is the lipid scramblase CED-8, homologous to mammalian Xkr8 (Y.-Z. Chen et al. 2013; Suzuki et al. 2016). Thus, acCED-3 initiates both apoptotic cell death and signaling for cell corpse clearance.

After PS externalization, the apoptotic cell needs to be recognized and engulfed. As *C. elegans* lacks migratory immune cells, neighboring cells engulf apoptotic cells. The secreted protein **t**rans**t**hyretin-**r**elated family domain TTR-52 binds to externalized PS and is recognized by the engulfment receptor CED-1 (X. Wang et al. 2010), which is a homolog of the mammalian MEGF10 protein (Hamon et al. 2006). CED-1 signaling via CED-6 (GULP) activates the dynamin DYN-1 to promote rearrangement of the actin cytoskeleton to from a phagocytic cup around the dying cell (Fig. 3) (Shen et al. 2013). Other receptors that recognize externalized PS include the **p**hosphatidyl**s**erine **r**eceptor PSR-1 (X. Wang 2003) and the heterodimer of **in**tegrin **α**1 INA-1/**p**aralyzed **a**rrest at **t**wofold integrin β PAT-3 (T.-Y. Hsu & Wu 2010). The receptor **m**ore **o**f **M**S MOM-5 is also important for recognition of dying cells, but it is not clear whether it binds PS or recognizes another signal on dying cells (Cabello et al. 2010). Together, CED-2, CED-5 and CED-12 promote the activation of the Rho family GTPase CED-10, homolog of mammalian Rac1 (Gumienny et al. 2001; Wu et al. 2001; Zhou et al. 2001; Brugnera et al. 2002; X. Wang 2003; H. Yang et al. 2014). CED-10 activation leads to actin rearrangement to form a phagocytic cup and engulf the apoptotic cell (see Fig. 3 (Kinchen et al. 2005).

Interestingly the engulfment process not only leads to cell clearance, but also contributes to cell death. Mutations in proteins involved in cell corpse engulfment, including *ced-1*, *ced-2*, *ced-6*, and *ced-10*, not only block the engulfment of dying cells, but also lead to a higher number of cells surviving embryogenesis. Some cells start the dying mechanism, but recover and survive (Hoeppner et al. 2001; Reddien et al. 2001). Ergo, cell engulfment not only re moves the cell, but also contributes to its death, known as phagoptosis (Brown & Neher 2012).

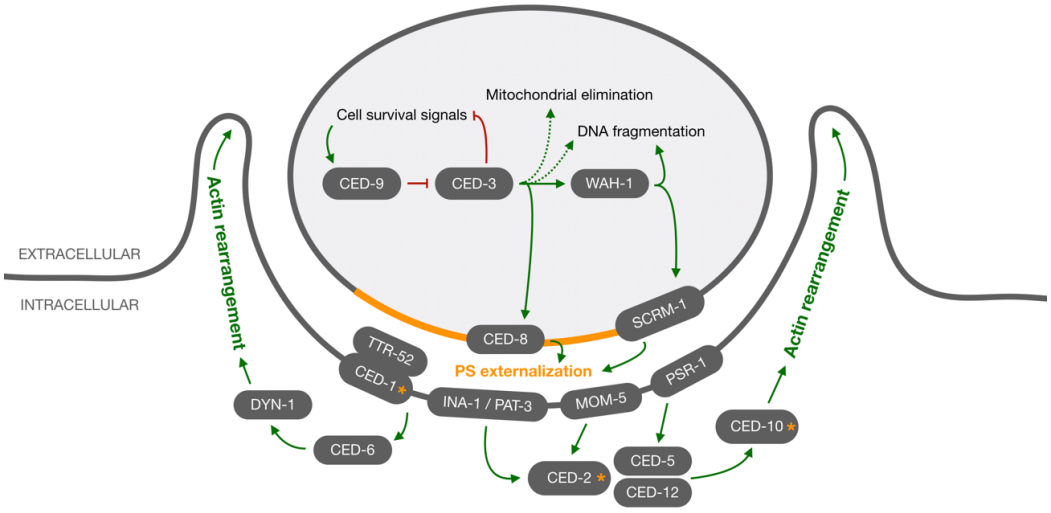


Figure 3 – Pathways leading to apoptosis and cell engulfment in *C. elegans*.
CED-9 is necessary for cell survival as it inhibits CED-3 caspase activity. Activation of CED-3 starts the apoptotic machinery, including mitochondrial elimination, the suppression of cell survival signals and DNA-fragmentation. WAH-1 and associated nucleases fragment DNA, but CED-3 cleavage of WAH-1 and CED-8 also leads to PS externalization to the outer leaflet of the plasma membrane which acts as an "eat-me" signal. CED-1 in the plasma membrane of neighboring cells recognizes externalized PS via secreted TTR-52. CED-1 then signals via CED-6 and DYN-1 to rearrange actin for cell engulfment. Other receptors that recognize PS include INA-1/PAT-3, MOM-5, and PSR-1, which directly (or indirectly via CED-2) activate CED-5 and CED-12. This will activate the Rac GTPase CED-10, which also leads to actin rearrangement. *Grey top cell is apoptotic cell, bottom cell is engulfing cell; * orange stars indicate proteins investigated in this work; green arrows show activating connections, red lines show inhibiting connections. Figure is based on Conradt (2016).*

1.5.2 Phagocytosis and phagosome maturation in *C. elegans*

Activation of the CED-1/MEGF10 receptor leads to DYN-1/dynamin activity, which in addition to regulating actin rearrangements for phagocytosis, is also thought to act as an initiating factor for phagosome maturation in *C. elegans* and mammals (Fig. 4). DYN-1 recruits the class III phosphatidylinositol 3-kinase (PI3K) VPS-34 and the Rab GTPase RAB-5 (Kinchen & Ravichandran 2008), which promote phagosome maturation by recruiting other maturation factors.

To mature the phagosome, proteins, including SAND-1 and CCZ-1, later promote RAB-5 dissociation from the phagosome (Kinchen & Ravichandran 2010). RAB-5 is exchanged for RAB-7 (Fig. 4), which promotes fusion with lysosomes (Harrison et al. 2003; Yu et al. 2008; Guo et al. 2010). At this point, the phagosome has already acidified with pH levels around 5 (McNeil et al. 1983).

Blocking RAB-5 release from the phagosome results in delayed RAB-7 recruitment, lysosome fusion and acidification (W. Li et al. 2009), underlining the importance of RAB-5 dissociation from the phagosome. RAB-7-driven lysosome fusion with the phagosome results in lysosome-associated membrane glycoprotein LMP-1 colocalization on phagosomes (Eskelinen et al. 2003). Further acidification and digestion by lysosomal proteins will degrade the phagocytosed cargo.

As the target for phagocytosis is recognized by receptors like CED-1 and engulfed, the receptors are also internalized on phagosomes (Fig. 4). Over time, this would result in continuously lower CED-1 receptor density at the plasma membrane and loss of the cell's phagocytosis function. The retromer complex counteracts CED-1 loss by recycling the receptor. The retromer complex promotes endosome-to-Golgi transport of transmembrane receptors and other proteins, both in worms and humans (Coudreuse et al. 2006; Verges 2007; Shi et al. 2009). The **s**orting **n**e**x**ins SNX-1 and SNX-6 associate with the retromer complex and have been shown to recycle CED-1 to the plasma membrane. Loss of either results in lysosomal degradation of CED-1 (Chen et al. 2010; Lu et al. 2011). SNX-1 and SNX-6 also physically interact with each other and have been shown to promote phagosome maturation, especially the fusion of phagosomes and intracellular vesicles (Lu et al. 2011).

Phagosome maturation is a complex yet well-choreographed process. The timely recruitment of each protein plays a crucial role in phagosome maturation, as defects can lead to serious disease (see section 1.1).

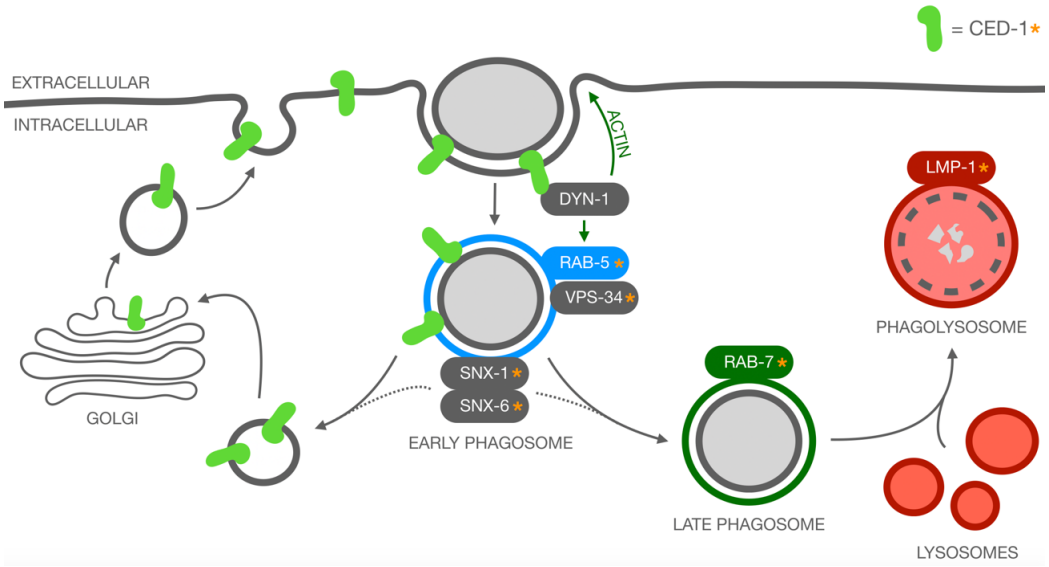


Figure 4 – Phagosome maturation and engulfment receptor recycling.
After engulfment, various factors are involved in phagosome maturation. DYN-1/dynamin leads to RAB-5 GTPase and VPS-34 PI3Kinase activity. RAB-5 quickly dissociates from the early phagosome and is exchanged for RAB-7. RAB-7 drives fusion with lysosomes for final degradation of the phagocytosed cargo. To recycle internalized CED-1 receptor, the sorting nexins SNX-1 and SNX-6 drive endosome to Golgi transport of CED-1. Both proteins also promote phagosome maturation.
*Grey cell is apoptotic cell; * orange stars indicate proteins investigated in this work; green arrow shows activating connection; dotted lines show involvement in processes.*

1.5.3 Death and clearance of *C. elegans* polar bodies

Polar bodies appear to be the first cells which die and need to be cleared during embryonic development of many organisms, including *C. elegans*. Meiosis and polar body extrusion in *C. elegans* happen concomitantly with eggshell formation, which leads to different positions and fates of the two polar bodies. The 1st polar body is extruded while the eggshell is forming and is therefore trapped between eggshell layers with no contact to embryonic cells (Olson et al. 2012). In contrast, the 2nd polar body is born after eggshell formation (Benenati et al. 2009). Thus, the 2nd polar body has direct contact to embryonic cells and could interact with the embryo. Furthermore, if the 2nd polar body becomes necrotic, it could endanger the developing embryo.

In *C. elegans,* both polar bodies undergo programmed cell death early in embryonic development, as their fragmented DNA can be stained using TUNEL (Wu et al. 2000). However, their death is independent of apoptotic caspases,

thus likely to be a non-apoptotic form of programmed cell death. An antibody that binds to engulfed apoptotic cells (F2-P3E3) also stains the second polar body, suggesting that it is internalized and/or disposed of by the same process as an apoptotic corpse (Eisenhut et al. 2005). Thus, the mechanisms of polar body death and clearance remain to be identified.

During their work on the clearance of midbody remnants by LC3-associated phagocytosis in *C. elegans* embryos (Fazeli et al. 2016), our lab observed that while the 1st polar body remained immobile in the egg shell, the 2nd polar body performed several stereotyped movements (see Fig. 5A). The polar bodies are extruded on the anterior side of the embryo, with the 2nd polar body located between the eggshell and the plasma membrane, where it is able to move between these two layers. During the AB cell division (3-cell stage), it often moved towards the anterior blastomere (AB) ingression furrow and in between the dividing AB cells. It was also internalized by one of the AB daughter cells before the embryo reached the 6-cell stage. A fluorescent PH domain reporter, which binds to phospholipids of the plasma membrane, clearly labeled the polar body after engulfment (Balla & Várnai 2009; Fazeli et al. 2018), suggesting that it was wrapped in a membrane after endocytosis by an unknown mechanism.

As the 2nd polar body is a relatively large extracellular object with an average diameter of 2.5 µm (Fazeli et al. 2018), it was likely to be internalized by phagocytosis (Flannagan et al. 2012). Phagocytosis requires actin rearrangement to form the large phagocytic cup (Chimini & Chavrier 2000). The lab had confirmed actin enrichment during engulfment by time-lapse imaging of worm strains expressing a fluorescent LifeAct marker, which binds to F-actin (Riedl et al. 2008; Fazeli et al. 2018). Before and until internalization, LifeAct accumulated around the 2nd polar body (see Fig. 5B), suggesting that actin rearrangements were occurring. Thus, the 2nd polar body appeared actively engulfed by the embryo, though it was open to be determined whether it was internalized by phagocytosis.

To confirm engulfment via phagocytic pathways, the lab disrupted CED-1 and CED-2 function in embryos using RNAi and observed that the 2nd polar

body was no longer internalized by the 6-cell stage (see Fig. 5C-D). Additionally, by staining with CED-1 antibody, they showed that the CED-1 receptor accumulated around the polar body before its internalization and remained on the polar body phagosome after engulfment (see Fig. 5E-F). Thus, the 2nd polar body appeared to be actively internalized via phagocytosis, depending on CED-1 and CED-2 function (Fazeli et al. 2018). However, there are more factors known to be involved in this process (see section 1.5.1), thus we needed to confirm their involvement and their regulation. Additionally, we knew nothing about the further phagosome maturation and degradation processes of the 2nd polar body, leaving these pathways open to be identified.

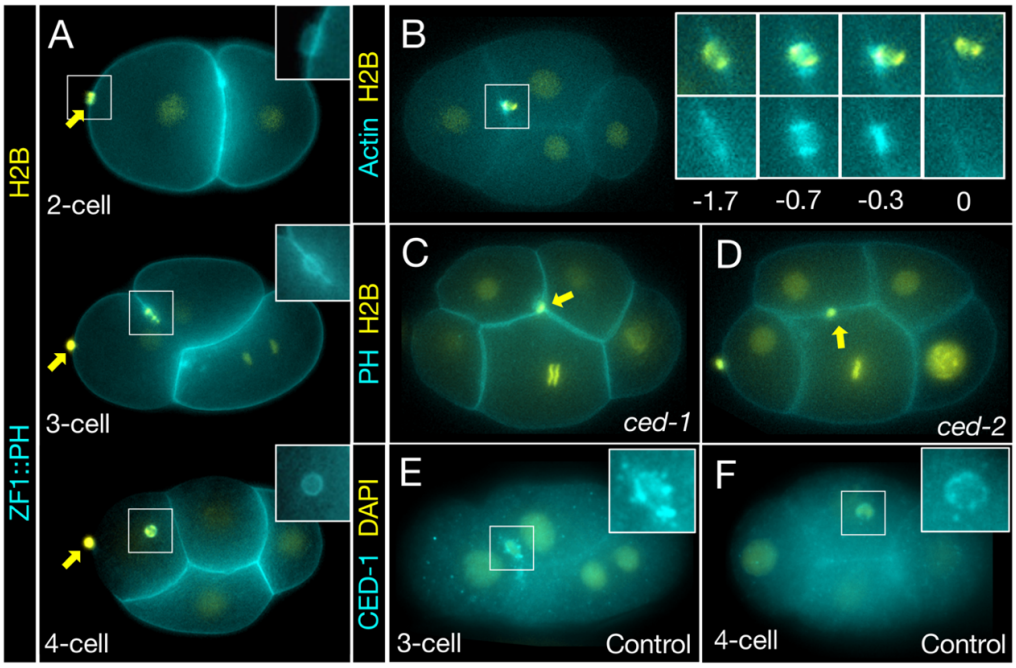


Figure 5 – The 2nd polar body is internalized via phagocytosis, dependent on CED-1 and CED-2.
(A) At the 2-cell stage, the 2nd polar body can be found at the cell surface of the anterior AB-cell. During the AB cell division, it is dragged in between the dividing AB cells. During the 4-cell stage, it is internalized by either ABa or ABp and can be found within a membrane in the cell. Yellow arrows indicate the 2nd polar body; embryo is expressing mCherry-tagged H2B to label nuclei and GFP-tagged PH to label membrane.
(B) Prior and until internalization, actin accumulates around the 2nd polar body. Numbers indicate time in minutes relative to internalization. Embryo is expressing mCherry::H2B to label nuclei and LifeAct::GFP to visualize F-actin.
(C-D) When depleted of either *ced-1* or *ced-2* by RNAi, the 2nd polar body is not internalized and can be found between embryonic cells in 6-cell stage embryos.
(E-F) In wild type embryos, the engulfment receptor CED-1 can be found to accumulate on the 2nd polar body before (E) and after (F) internalization. Embryos were stained with CED-1 antibody and DAPI to label nuclei.
Images were published in Fazeli, et al., Cell Reports 2018.

1.6 Aims of this work

In this work, I characterize the mechanisms of polar body death and clearance using *C. elegans* as a model organism. As mammalian polar bodies appear to become necrotic, I first investigated the nature of polar body death in *C. elegans*, specifically exploring whether they would become necrotic *in vivo* and therefore pose a potential threat to the developing embryo. To characterize how the 2nd polar body interacts with the embryo and successfully signals for its internalization, I characterized the timing of internalization of the 2nd polar body using time lapse imaging *in vivo*. Using genetic approaches, I also identified factors regulating polar body internalization and movement and placed them in a molecular pathway. I characterized the maturation of the polar body phagosome in order to understand the highly choreographed process of corpse clearance, including the timely recruitment of maturation factors and lysosome fusion. Finally, I identified factors influencing maturation, including proteins involved in LC3-associated phagocytosis. This work establishes the *C. elegans* polar body as a genetic model to study non-apoptotic cell death, engulfment, phagosome maturation and resolution by LC3-associated phagocytosis. This new model system will enable further research to improve our understanding of these dynamic processes *in vivo*, possibly linking them to diseases and allowing the development of novel therapies for autoimmune, degenerative and other diseases.

2 MATERIALS AND METHODS

2.1 Worm strains

All *C. elegans* strains were maintained at room temperature and handled according to standard protocols (Brenner 1974). Strains used in this work are listed in Table 1.

Strain	Genotype	Source
N2	Wild type	(Brenner 1974)
AZ212	*unc-119(ed3) ruIs32[pAZ132: pie-1p::GFP::H2B; unc-119(+)] III*	(Praitis et al. 2001)
BV67	*zbIs1[pie-1p::Lifeact::GFP; unc-119(+)]; ltIs44[pie-1p::mCherry::PH(PLC1delta1); unc-119(+)] V.*	(Pohl & Bao 2010)
BV113	*zuIs45[nmy-2::NMY-2::GFP + unc-119(+)] IV; zbIs2 [pie-1::lifeact-Cherry + unc-119(+)]*	(Singh & Pohl 2014)
CB3203	*ced-1(e1735) I*	(Horvitz et al. 1983)
GK682	*unc-119(ed3) III; dkIs398[Ppie-1::GFP::lgg-1; unc-119(+)]*	(M. Sato & K. Sato 2011)
HT1593	*unc-119(ed3) III*	(Davis et al. 2009)
OD70	*unc-119(ed3) III; ltIs44[pie-1p::mCherry::PH(PLC1∂1); unc-119(+)] V*	(Kachur et al. 2008)
RT122	*unc-119(ed3) III; pwIs20[pie-1p::gfp-rab-5; unc-119(+)]*	(M. Sato et al. 2005)
RT123	*unc-119(ed3) III; pwIs21[pie-1p::gfp-rab-7; unc-119(+)]*	(Kang et al. 2011)
RT1952	*unc-119(ed3) III; pwIs700[pID3.01-snx-1: pie-1p::GFP::snx-1; unc-119(+)]*	(Fazeli et al. 2018)
VIG25	*Is[pVIG57: Pmex-5::mCherry::LGG-2::tbb-2 3'UTR; C.b. unc-119(+)] II; unc-119(ed3) III*	(Fazeli et al. 2018)
WEH51	*unc-119(ed3) III; xnIs65 (nmy-2-gfp-zf1, unc-119) IV; ltIs44 [pie-1p-mCherry::PH(PLC1delta1)] V*	(Fazeli et al. 2016)
WEH55	*vps-34(h510) dpy-5(e61) I; ltIs38[pie-1p::GFP::PH(PLC1∂1); unc-119(+)] xnIs8[pJN343: nmy-2::NMY-2::mCherry; unc-119(+)] unc-119(ed3) III; enEx441[vps-34(+); ced-1C::mRFP]*	(Fazeli et al., 2016)
WEH61	*ced-1(e1735) I; xnIs65 (nmy-2-gfp-zf1, unc-119) IV; ltIs44 [pie-1p-mCherry::PH(PLC1delta1)] V*	(Fazeli et al. 2016)
WEH69	*bec-1(ok691) xnIs65[nmy-2-gfp-zf1, unc-119] / nT1 IV; ltIs44 [pie-1p-mCherry::PH(PLC1delta1)] / nT1[qIs51] V*	(Fazeli et al. 2016)
WEH73	*vps-34(h510) dpy-5(e61) I; unc-119(ed3) III; xnIs65[nmy-2::gfp::zf1; unc-119(+)] IV; ltIs44[pie-1p::mCherry::PH(PLC1∂1); unc-119(+)] V; enEx441[vps-34(+); ced-1C::mRFP]*	(Fazeli et al., 2016)
WEH78	*ltIs38[pie-1::GFP::PH(PLC1delta1) unc-119(+)] xnIs8 [pJN343: nmy-2::NMY-2-mCherry; unc-119(+)] unc-119(ed3) III; ced-2(e1752) IV*	(Fazeli et al. 2016)
WEH85	*ced-2(e1752) zuIs45 [nmy-2::NMY-2::GFP + unc-119(+)] IV; zbIs2 [pie-1::lifeACT::RFP + unc-119(+)]*	(Fazeli et al. 2016)
WEH71	*ced-2(e1752) xnIs65[nmy-2-gfp-zf1, unc-119] IV; ltIs44 [pie-1p-mCherry::PH(PLC1delta1)] V*	(Fazeli et al. 2016)
WEH95	*unc-119(ed3) III; pie-1p::mCherry::HistoneH2B IV; xnIs390[pie-1p::GFP::ZF1::PH(PLC1delta1), unc-119(+)]*	(Beer et al. 2018)

Strain	Genotype	Source
WEH139	*unc-119(ed3) III; pie-1p::mCherry::HistoneH2B IV*	Crossed N2 to WEH95
WEH141	*rrf-1(pk1417) I; unc-119(ed3) III; xnIs65 [nmy-2:: gfp::zf1 + unc-119(+)] IV; ltIs44 [pie-1p-mCherry:: PH(PLC1ϑ1) + unc-119(+)] V*	(Fazeli et al. 2016)
WEH142	*unc-119(ed3) III; zbIs1[pie-1p::Lifeact-GFP; unc-119(+)]; pie-1p::mCherry-HistoneH2B IV*	Crossed WEH139 to BV67
WEH225	*Is[pVIG57: mex-5p::mCherry::LGG-2::tbb-2 3'UTR, C.b. unc-119(+)] II; unc-119(ed3) ruIs32[pAZ132: pie-1p::GFP::H2B, unc-119(+)] III*	Crossed AZ212 to VIG25
WEH231	*unc-119(ed3) ruIs32[pAZ132: pie-1p::GFP::H2B, unc-119(+)] III; ltIs44[pie-1p-mCherry::PH(PLC1delta1), unc-119(+)] V*	Crossed OD70 to AZ212
WEH235	*unc-119(ed3) ruIs32[pAZ132: pie-1p::GFP::H2B, unc-119(+)] III; pie-1p::mCherry::HistoneH2B IV*	Crossed WEH139 to AZ212
WEH246	*unc-119(ed3) III; pwIs20[pie-1p::gfp::rab-5; unc-119(+)]; pie-1p::mCherry::HistoneH2B IV*	Crossed WEH139 to RT122
WEH248	*unc-119(ed3) III; pwIs21[pie-1p::gfp::rab-7, unc-119(+)]; pie-1p::mCherry::HistoneH2B IV*	Crossed WEH139 to RT123
WEH260	*unc-119(ed3) III; wurIs90[pGF7:pie-1p::mCh::PH::ZF1, unc-119(+)]*	(Beer et al. 2018)
WEH271	*unc-119(ed3) III; wurIs96[pGF08:pie-1::mKate2::ZF1::npp-5; pJN254:unc-119(+)]*	(Fazeli et al. 2018)
WEH295	*unc-119(ed3) III; pie-1p::mCherry::HistoneH2B IV; pwIs700[pID3.01-snx-1: pie-1p::GFP::snx-1; unc-119(+)]*	Crossed WEH139 to RT1952
WEH296	*unc-119(ed3) III; +/wurIs103[pGF13: pie-1::ZF1::mCherry::his-15; unc-119(+)]*	(Fazeli et al. 2018)
WEH304	*unc-119(ed3) III; wurEx14[lmp-1::GFP fosmid WRM0636B_G02]*	Bombarded HT1593
WEH306	*unc-119(ed3) III; pie-1p::mCherry::HistoneH2B IV; wurEx14[lmp-1::GFP fosmid WRM0636B_G02]*	Crossed WEH139 to WEH304
WEH336	*unc-119(ed3) III; pie-1p::mCherry-HistoneH2B IV; dkIs398[Ppie-1::GFP::lgg-1; unc-119(+)]*	Crossed WEH139 to GK682
WEH372	*unc-119(ed3) III; [mCherry::HistoneH2B] IV; wurIs125[pie-1::ssGFP::C2(mfge8), unc-119(+)]*	(Fazeli et al. 2018)
WEH382	*epg-8(ok2561) / hT2[bli-4(e937) let-?(q782) qIs48] I; Is[pVIG57: Pmex-5::mCherry::LGG-2::tbb-2 3'UTR, C.b. unc-119(+)] II; unc-119(ed3) ruIs32[pAZ132: pie-1::GFP::H2B, unc-119(+)] / hT2 III*	Crossed N2 to WEH225 and then to WEH275

Table 1 Worm strains used in this work, their genotype and source.

2.1.1 Crossing worm strains

The worm strains WEH142, WEH225, WEH231, WEH235, WEH246, WEH248, WEH260, WEH295, WEH306 and WEH336 were created by crossing

two existing strains with each other, in order to get two different fluorescent markers expressed in one strain.

To cross two strains, L3-L4 worms of one strain were heat-shocked at 33°C for 4 hours to generate males. Male progeny was picked three days later onto a crossing plate with L4 hermaphrodites from the other strain. The hermaphrodites (F_0-generation) were separated the next day and their progeny (F_1-generation) was screened for males two days later. If there were any males (indicating a successful mating), their hermaphrodite siblings were picked onto a fresh plate to self-fertilize, as all were expected to be heterozygous for both markers. The F_2-generation was then singled onto two 24-well plates. According to Mendelian inheritance, 1/16 of this generation is expected to be homozygous for both markers. To find the homozygous strain, the F_3-generation was screened for expression of both fluorescent markers. When at least 20 out of 20 worms expressed both markers, the worm strain can be expected to be homozygous (with a probability > 99.99%).

2.1.2 Worm transformation

WEH304 was created by microparticle bombardment of HT1593 using standard protocols (Schweinsberg & Grant 2013) with the following GFP-tagged LMP-1 fosmid from the TransgeneOme project (Sarov et al. 2012): WRM0636B_G02(pRedFlp-Hgr)(lmp-1[21661]::S0001_-pR6K_Amp_2xTY1ce_EGFP_FRT_rpsl_neo_FRT_3xFlag)dFRT::unc-119-Nat

2.1.3 WEH306 maintenance

The LMP-1::GFP reporter in WEH304 and WEH306 is on an extrachromosomal array, which is inherited by approximately two-thirds of the progeny. To keep the marker expressed in WEH306, GFP-positive worms were selected manually. This was performed using an Olympus SZX16 stereo microscope equipped with a U-HGLGPS fluorescent light source. It was possible to pick positive worms, let them starve and chunk them up until two months later, still finding GFP-expressing worms. Freezing worms, however, made them lose the LMP-1::GFP reporter.

2.2 RNAi experiments

RNAi was primarily performed by bleaching gravid adults onto RNAi plates and therefore feeding their larval progeny at 25°C for 60-70 hours until adulthood. RNAi plates contained bacteria expressing dsRNA plasmids on NGM Lite agar supplemented with Ampicillin, Tetracycline, and Lactose following established standard protocols (Fraser et al. 2000). All RNAi constructs were obtained from available libraries (Source BioScience). The following clones were used: *ced-10* (mv_C09G12.8), *ced-1* (mv_Y47H9C.4)*, ced-2* (mv_G_YK2769*), rab-7* (mv_W03C9.3)*, atg-7* (sjj_M7.5), *ptr-2* (sjj_C32E8.8) and *snx-6* (mv_Y59A8B.22).

WEH231 was fed for 80-90 hours at 25°C, because their generation time was roughly four days, possibly due to an unknown background mutation.

For *rab-7* RNAi, L4 worms were picked, washed and treated with RNAi for 18-25 hours because *rab-7* RNAi resulted in lethality when worms were treated for three days.

For *ptr-2* RNAi, L4 worms were treated for 14-20 hours at 20°C to give the best results in terms of normal polar body extrusion and polarity of the embryo, as described by (Carvalho et al. 2011).

2.3 Imaging

For live imaging, adult worms were picked into M9 buffer (3.0 g KH2PO4, 6.0 g Na2HPO4, 0.5 g NaCl, 1.0 g NH4Cl, per liter of H_2O) on a cover slip and dissected with a scalpel to release the embryos in the buffer. The cover slip was then put on a slide with an agarose pad. The space between the cover slip and the slide was filled with M9 buffer and sealed with Vaseline to prevent the embryos from drying out during time-lapse data collection.

For imaging, a LEICA DM5500 wide-field fluorescence microscope was used, equipped with a LEICA DFC365 CCD camera controlled by LAS AF software. GFP and mCherry were captured sequentially in a 16 Z-stack with 1,2 µm distance between each stack. For time-lapse imaging, the time between each z-

stack was either 20 seconds or 1 minute. For individual pictures, the Z-stack had various sizes. All imaging was conducted under controlled room temperature.

2.4 Antibody staining

Gravid adults were picked, washed in water and then dissected in water on a coverslip with 0,1% polylysine followed by immediate freezing on dry ice. The eggshell was cracked by flicking off the coverslip before fixing the embryos in methanol. After fixation, embryos were stained with mouse α-CED-1 antibody (1:500, gift of Chonglin Yang). DNA was stained using DAPI and the slide was mounted using DABCO and sealed with nail polish. For imaging details see section 2.3.

2.5 Propidium iodide staining

Gravid adults were picked and dissected in Embryonic Culture Medium (ECM; 12µl Penn-Strep solution (Gibco 3353), 900 µl L15 culture medium, 100 µl Fetal Calf Serum (heat treated), 50 µl 20% sucrose) or in egg salts (118 mM NaCl, 40 mM KCl, 3.4 mM MgCl2, 3.4 mM CaCl2, 5 mM HEPES pH 7.4) to release the embryos into the buffer. We tried various buffer solutions, because we made the eggshell permeable for this experiment, exposing embryonic cells to the surrounding medium. Using ECM gave the highest rate of healthy embryos (compared to M9 and egg salts), as it is the closest to the embryo's osmolarity. Embryos were transferred in 2µg/ml propidium iodide solution (ThermoFisher P1304MP, provided by Bernhard Nieswandt) and incubated for 15 minutes in the dark. The embryos were mounted on an agarose pad on a microscope slide. For imaging details see section 2.3.

2.6 Analysis of imaging data

Time lapse data and still images were analyzed using Imaris by Bitplane. The following data was manually annotated.

Cell stage timing: Each cell stage is defined by the first visible ingression of the dividing cell's plasma membrane.

Analysis of the stereotyped interblastomeric movement of the 2nd polar body: Embryos were scored for the stereotyped movement of the 2nd polar body towards the ingression furrow of the anterior blastomere (AB cell) and whether the polar body was found in between the newly born AB daughter cells.

Internalization time: The time of internalization is defined as the first frame when the polar body moves away from the cell membrane into the cell. This highly correlates with actin polymerization at the scission site and therefore is likely to reflect the closure of the phagocytic ring (see section 3.3.1).

Colocalization of a fluorescent marker with the polar body marker: Colocalization between the polar body and another marker was noted when the polar body is trackable in time lapse data with the colocalization marker without the specific polar body marker. Colors were changed so that the polar body marker appeared yellow and the colocalization marker in cyan, allowing a better visibility of the markers. In still images, the colocalization was marked positive whenever there was a clear increase of the colocalization marker on the polar body.

Colocalization of LMP-1 with the polar body marker: The colocalization of a polar body marker with the lysosome-associated membrane protein (LAMP) homolog LMP-1 was considered positive whenever the intensity of LMP-1 around the 2nd polar body was higher than the florescence intensity of the marker on the plasma membrane of the embryo. This ensures that colocalization was only scored after an increase of LMP-1 on the polar body and was not caused by the fluorescence of the phagocytic membrane.

Colocalization of LGG-1/-2 and the polar body marker in time lapse series and pictures: As LGG-1/-2 is also expressed in the cytoplasm of the 2nd polar body and the embryo, colocalization was marked positive when LGG-1/-2 levels on the 2nd polar body were higher than the surrounding cytoplasm. This ensures that colocalization is due to an increased presence of LGG-1/-2 and not to fluorescence background in the cytoplasm.

Degradation timing: The time of degradation is defined as the first frame when the second polar body is not visible or not differentiable from the surrounding markers or background noise. As small fluorescent remains of the 2nd polar

body sometimes disappear and reappear between two Z-stacks, the following 5 frames were also screened for fluorescent puncta.

2.7 Image manipulation

Images were rotated, color intensity was adjusted, and the embryos were colorized in yellow and cyan by using Adobe Photoshop to optimize visibility. All images show only one Z-section, except for Fig. 11D, which shows two sections max projected on each other.

2.8 Statistics

For statistical analysis, Student's t-test and Fisher's exact test were used. Statistical method is indicated in the text or figure legends. For multiple comparisons, we used the Bonferroni correction. Mean and standard deviation (SD) is reported in the text. Figure 10A shows Mean ± standard deviation (SD), while other figures show mean ± standard error of the mean (SEM), as indicated in the figure legend.

3 RESULTS

3.1 Death of the polar bodies

Dying cells pose a threat to neighboring cells and to the whole organism, as they can induce inflammation (Martinez et al. 2016; Poon et al. 2014). Similarly, dying polar bodies could pose a threat to the developing embryo, leading to a stress response of embryonic cells. This would especially be the case if the 2nd polar body loses membrane integrity. Determining the mechanism of cell death could also elucidate why the 2nd polar body is cleared by the developing embryo. Therefore, it is important to understand how polar bodies die and to determine whether they die before internalization.

3.1.1 Polar bodies become necrotic

Given that the 2nd polar body undergoes non-apoptotic programmed cell death in *C. elegans* (Wu et al. 2000) and is internalized by the embryo, we asked whether polar bodies become necrotic. As necrotic cells lose membrane integrity (Elmore 2007), we stained embryos with propidium iodide (PI), a nuclear stain that is not able to cross intact membranes. Loss of membrane integrity allows PI to label necrotic cells. Healthy or apoptotic cells cannot be penetrated by PI as their membrane remains intact (Vlachos & Tavernarakis 2010).

We stained embryos expressing a fluorescent H2B reporter to label histones in nuclei, including the polar body nuclei. In all cases (n=55 embryos), the 1st polar body was stained by PI as early as the 1-cell stage. This observation indicates that the 1st polar body loses membrane integrity quickly after its extrusion, becoming necrotic soon after meiosis (see Fig. 6A).

In contrast to the 1st polar body, the 2nd polar body was not stained by PI (n=0/10) in early cell stages (2-cell to 12-cell stage). Since the 2nd polar body is quickly internalized after its extrusion, we hypothesized that it may be internalized before it loses membrane integrity. To test for that, we depleted CED-2 to prevent polar body internalization, giving PI more time to stain the 2nd polar body. First, we checked whether the 1st polar body was still stained. Out of 25 *ced-2* knockdown embryos, 24 1st polar bodies were positive for PI, the earliest of

which was at the pronuclear or pseudocleavage stage. Thus, PI staining of the 1st polar body was not affected by *ced-2* knockdown and the 1st polar body becomes necrotic before the first mitotic cell division. One embryo was photographed during Meiosis II with a PI-negative 1st polar body, suggesting that loss of membrane integrity happens between Meiosis II and pronuclear meeting (a 10-minute window). Given the timing of Meiosis I, when the 1st polar body is extruded (McCarter 1998), the 1st polar body dies 15 to 30 minutes after its birth.

As the 1st polar body was still necrotic after *ced-2* knockdown, we next examined the 2nd polar body. In contrast to the 1st polar body, there were no positive 2nd polar bodies after *ced-2* knockdown (n=0/18, see Fig. 6B). Thus, preventing internalization of the 2nd polar body still did not allow PI to stain it. This left open two possibilities: either the 2nd polar body does not become necrotic or there is another barrier for PI around the 2nd polar body.

In contrast to the 1st polar body, the 2nd polar body is born after eggshell formation (Fabritius et al. 2011; Olson et al. 2012). The eggshell includes layers that forms a permeability barrier for the embryo, which are known to lie between the 1st and the 2nd polar body (Olson et al. 2012). Therefore, we hypothesized that this eggshell layer could prevent PI from staining the cells inside it, including the 2nd polar body. To make the eggshell permeable for PI, worms were depleted of PTR-2 by RNAi. PTR-2 is required for secretion of the permeability barrier (Carvalho et al. 2011). After *ptr-2* RNAi treatment, both polar bodies were stained by PI (see Fig. 6C-D). The 1st polar body was PI-positive as early as the pronuclear/pseudocleavage stage (n=3/3), similar to results in wild type and *ced-2* knockdown embryos. The earliest PI-positive 2nd polar body was found in 1-cell stage embryos (n=1/7), over 20 minutes after its extrusion and 20 to 40 minutes before it is internalized. Including embryos up until the 24-cell stage, 39% of 2nd polar bodies lost their membrane integrity (n=24/61). Therefore, the eggshell forms a permeability barrier around the 2nd polar body that PI is unable to penetrate. Disrupting this barrier reveals that both polar bodies become necrotic during early embryonic development. This revelation underscores the importance

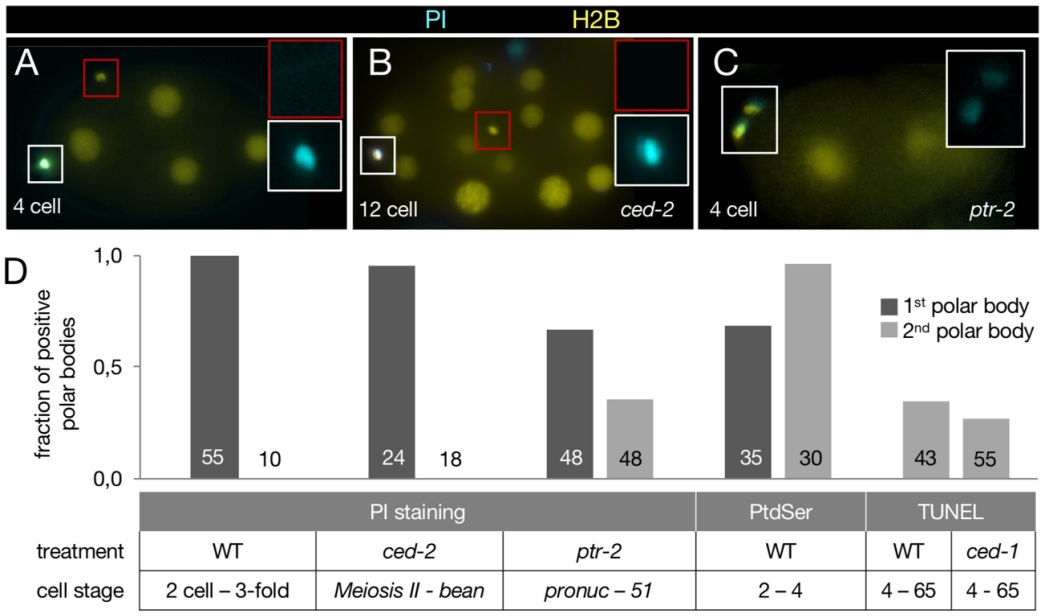
of clearing the 2nd polar body, as its loss of membrane integrity presents a potential risk for the neighboring embryo.

Depletion of PTR-2 also resulted in defects in polar body positioning, as previously reported (Carvalho et al. 2011). Some embryos did not extrude their polar bodies on the anterior side and the 1st polar body was no longer trapped in the eggshell, but was positioned next to the embryo, similar to the 2nd polar body. Thus, it was only possible to distinguish the two polar bodies based on their different histone content. PTR-2 depletion also allowed three 1st polar bodies to be internalized by the embryo (n=3/69), suggesting that the eggshell barrier normally prevents internalization of the 1st polar body. However, PTR-2 depletion also lowered internalization rates of the 2nd polar body after the 4-cell stage to 59% (n=17/30), possibly since the eggshell supports internalization by increasing the contact between the polar body and embryonic cells. Furthermore, PI stained 1st polar bodies visibly weaker after PTR-2 depletion than in untreated or CED-2 depleted embryos. The stained 2nd polar bodies also showed weak PI staining, but the cause for weaker staining after PTR-2 depletion remains to be investigated.

To further characterize the mechanism of polar body death, colleagues in the lab tested whether the death of the 2nd polar body depended on its phagocytosis by the embryo. They tested this using TUNEL-staining, which stains fragmented DNA (Kyrylkova et al. 2012) and was previously shown to label the 2nd polar body (Wu et al. 2000). They discovered that the 2nd polar body became TUNEL-positive in *ced-1* mutant embryos, despite not being engulfed (see Fig. 6D). They found TUNEL-positive 2nd polar bodies as early as the 4-cell stage in both wild type and *ced-1* mutant embryos. This data confirms the PI data showing that the 2nd polar body undergoes rapid cell death independent of phagocytosis.

They also looked for potential signals a dying polar body could send that would lead to its engulfment. They found that both polar bodies externalize the lipid phosphatidylserine as early as the 2-cell stage (PtdSer; see Fig. 6D). Comparing these data to the timing of PI staining suggests that both polar bodies

lose their membrane integrity before they signal for internalization. As the 1st polar body is in the eggshell and does not have contact to embryonic cells, this data also suggests that “eat-me” signals are presented autonomously by the polar bodies. These findings confirm that polar bodies undergo programmed necrosis during early embryonic development, independent of their surrounding cells.



	PI staining			PtdSer	TUNEL	
treatment	WT	*ced-2*	*ptr-2*	WT	WT	*ced-1*
cell stage	2 cell – 3-fold	*Meiosis II - bean*	*pronuc – 51*	2 – 4	4 – 65	4 - 65

Figure 6 – Dying polar bodies lose membrane integrity.
(A) The 1st polar body (white square) is stained by propidium iodide (PI), while the 2nd polar body (red square) is negative for PI, although it is not yet internalized.
(B) After disrupting internalization by *ced-2*-RNAi, the 2nd polar body is still PI-negative at later stages.
(C) After making the eggshell permeable using *ptr-2*-RNAi, both polar bodies are stained weakly by PI. Since they are both found next to the embryo, they are only distinguishable by their DNA content. The upper polar body is likely to be the 2nd polar body due to its lower H2B and PI fluorescence.
(D) Graph showing the fraction of positive 1st and 2nd polar bodies after PI-staining and various RNAi-treatments. Phosphatidylserine (PtdSer) was found to be externalized on both the 1st and the 2nd polar body during early embryonic development. TUNEL stained 2nd polar bodies in both untreated embryos and in embryos with disrupted phagocytosis due to *ced-1* RNAi treatment. PtdSer and TUNEL data were provided by Jaime N. Lisack. *Numbers on graph indicate n, PtdSer=phosphatidylserine, WT=wildtype, pronuc=pronuclear stage.*
Panel A has been published in Fazeli, G. et al., CellReports, 2018.

3.1.2 PI also stains differentiating endodermal precursor cells

Surprisingly, depleting PTR-2 to make the eggshell permeable also resulted in PI staining the cytoplasm of living embryonic cells in older embryos (Fig. 7). This was observed from the 48-cell stage up until the comma stage

(n=32). Staining was observed in a connected group of cells, mostly reaching from the center to the posterior section of the embryos. Cells in this area are most likely derived from the E cells (endodermal precursors). However, identifying the polarity of the embryo was sometimes difficult as the localization of the 1st polar body and the overall shape of the embryo were often disrupted after *ptr-2* knockdown. It is therefore possible that differentiating E cells somehow lose plasma membrane integrity, as they are stained by PI. However, unlike necrotic cells, PI mainly stains the cytoplasm of these cells There is no increase of PI seen in the nucleus of these cells. PI is known to bind RNA (Wallen et al. 1982), which means the PI staining may indicate RNA in the cytoplasm of the endoderm cells. How PI accesses the endoderm cytoplasm is unclear.

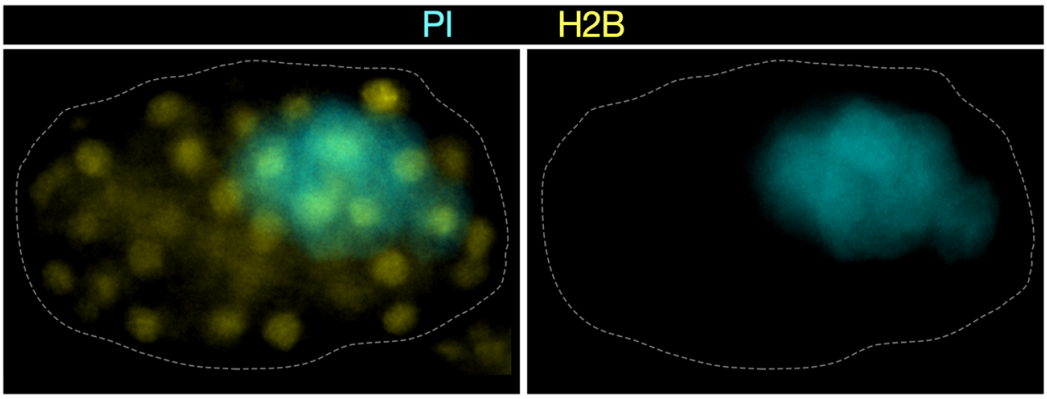


Figure 7 – PI stains living cells during early embryonic development, likely endodermal cells.
A section of cells in the posterior segment of the embryo has been stained by PI after PTR-2 depletion. The PI staining fills the whole cell.

3.2 The fate of the 1st polar body

As the 1st and the 2nd polar bodies appear to die in similar ways, but are located in different positions during embryonic development, we wondered if the 1st polar body persists until the worm hatches from its eggshell. To confirm that the 1st polar body is trapped in the eggshell throughout embryogenesis, I made a time lapse movie of embryogenesis until hatching with a strain that expressed

fluorescent H2B marker to label histones in nuclei, including the polar body nuclei. This also allowed me to test whether the contents of the 1st polar body are degraded after losing membrane integrity.

The movie showed that the 1st polar body persists in the eggshell during embryogenesis and remains stationary until hatching. After hatching, the 1st polar body was visible in the remains of the eggshell near the larva (see Fig. 8 and Video 1). The GFP::H2B marker was still visible after hatching, suggesting that at least some of the proteins in the 1st polar body are not degraded, acidified, or otherwise lost before hatching. This suggests that the eggshell provides a barrier around the necrotic 1st polar body, despite its loss of membrane integrity visible by PI staining.

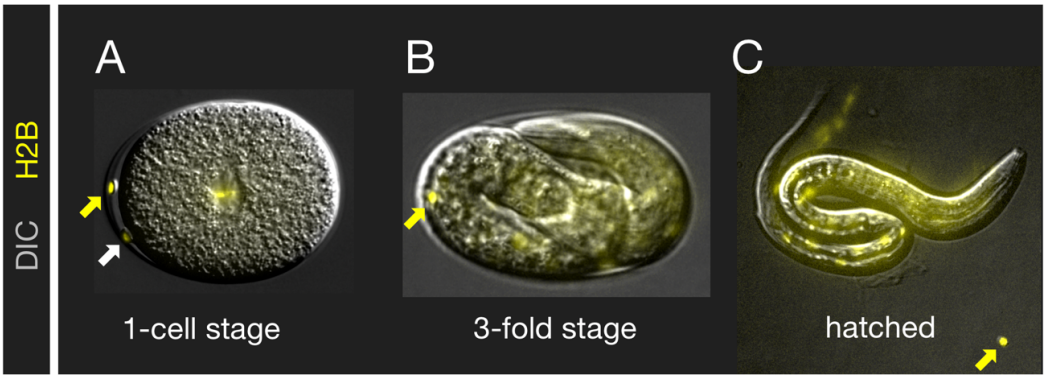


Figure 8 - The 1st polar body persists in the eggshell until hatching.
(A) The 1st polar body (yellow arrow) is found in eggshell layers on the anterior side of the embryo. The nearby 2nd polar body (white arrow) is located between the eggshell and embryo's plasma membrane.
(B) At the 3-fold stage (~9 hours after the 1-cell stage), the 1st polar body is still found in eggshell layers on the anterior side.
(C) The 1st polar body can be found within remains of the eggshell next to the hatching L1 larva (~11 hours after the 1-cell stage). The 1st polar body still expresses the GFP-tagged H2B reporter.
Panel C has been published in Fazeli, G. et al., CellReports, 2018.

Video 1 – Time lapse of embryogenesis until hatching.
The 1st polar body (arrow) is trapped in eggshell layers on the anterior side of the embryo. After hatching, the 1st polar body is found next to the hatched larva. The time lapse shows one frame every 10 minutes. Go to t1p.de/1stpb or scan QR code.
This video has been published in Fazeli, G. et al., CellReports, 2018.

3.3 The internalization of the 2nd polar body

As the 2nd polar body is positioned next to the embryo's plasma membrane, its loss of membrane integrity causes a potential risk for the embryo. Therefore, clearance of the 2nd polar body is an important process that needs to

be well choreographed. I analyzed the timing, mechanism as well as regulatory factors of internalization and the movement of the 2nd polar body prior to internalization to better understand how the embryo deals with a necrotic cell during early embryonic development.

3.3.1 The internalization movement highly correlates with the end of actin accumulation

To determine how stereotyped the engulfment of the 2nd polar body is, we first needed to define the criteria to score the internalization time point. Colleagues in the lab had already observed actin accumulation around the 2nd polar body during its internalization and showed that the 2nd polar body is actively internalized via phagocytosis. Actin polymerization is known to drive the extension of the phagocytic cup (Chimini & Chavrier 2000) and is therefore likely to represent the exact time point of polar body internalization. We asked whether the peak of actin enrichment correlated with polar body movement into the cell, as it does for midbody remnants phagocytosed by early embryonic cells (Fazeli et al. 2016).

I analyzed time lapse movies of WEH142, a strain that expresses fluorescent mCherry::H2B to label chromosomes (including in the polar bodies) and a fluorescent GFP::LifeAct actin reporter. I compared the timing of 2nd polar body movement into the cell with the timing of the end of actin accumulation on the polar body. The average difference between both time points was 30 ± 49 seconds (see Fig. 9). The coefficient of determination is 0,98 R^2, indicating a high correlation between both time points. Therefore, we decided to use the movement of the 2nd polar body into the internalizing cell as the internalization time point, as it highly correlates with the closure of the phagocytic ring and therefore likely represents the internalization time. Additionally, it allowed us to score for internalization time points in worm strains which are not expressing an actin marker.

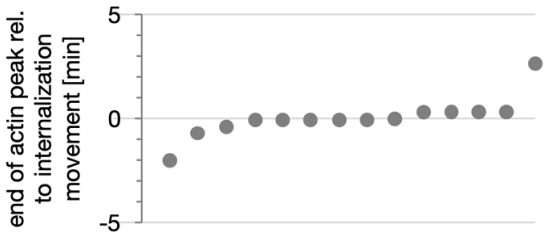


Figure 9 – The internalization movement highly correlates with the end of actin accumulation. Dot graph shows the time of the end of actin accumulation on the 2nd polar body relative to its movement into the embryo cytoplasm. There is a high correlation between these two time points with a coefficient of determination of 0.98 R^2. (n=14)

3.3.2 The 2nd polar body is internalized in a stereotyped manner

To characterize how embryonic cells interact with the 2nd polar body, we analyzed the timing and pattern of polar body internalization. Time lapse movies were made using worm strains expressing different fluorescent reporters. We analyzed strains that express a fluorescent H2B marker to label the chromosomes of the polar body (strain WEH142), a fluorescent PH reporter that binds to phospholipids in the plasma membrane of the 2nd polar body, as well as the membrane of embryonic cells (WEH260), a fluorescent NPP-5 reporter to label the nuclear membrane (WEH271) or fluorescent HIS-15 which labels H2B as well (WEH296). The cell membrane was either visualized by Differential interference contrast microscopy (DIC; WEH271, WEH296) or by using a fluorescent LifeAct Actin reporter (WEH142) that binds to the cell cortex (Doherty & McMahon 2008). Time lapse data for WEH260, WEH271 and WEH296 were captured and analyzed by Jaime Lisack and included in my cumulative analysis.

Consistent with the lab's preliminary findings, the 2nd polar body is internalized by the embryo before the 6-cell stage in nearly all embryos examined (98%, n=85/87). The internalization of the 2nd polar body happened during the 2-cell stage in 27% of embryos (n=24/87) or during the 4-cell stage in 70% of embryos (n=61/87; see Fig. 10A). As internalization after the 4-cell stage rarely happened (n=2/87), this was categorized as a delay or failure in internalization. Intriguingly, no internalizations occurred during the few minutes of the 3-cell stage (Fig. 10A). Indeed, there was a 5-minute gap between the internalizations

at the 2- and 4-cell stages that correlated with the timing of the cytokinetic ring during AB cell division (3-cell stage), suggesting cytokinesis blocks internalization of the second polar body, possibly since actin is required for closing the contractile ring of the dividing cells.

I tested whether there is a significant difference between the number of internalized polar bodies in 2- and 4-cell stage using Fisher's exact test and found no significant difference between all worm strains ($p>0.05$). I next asked whether there is a significant difference between the timing of internalization between the worm strains analyzed. When internalization happened during the 4-cell stage, the average internalization time after the 3-cell stage was 6:35 ± 1:20 min (n=27) in WEH142, 7:10 ± 1:20 min (n=9) in WEH260, 6:10 min ± 1:20 min (n=8) in WEH271, 7 ± 0:50 min (n=7) in WEH296, all not being significantly different from each other ($p>0.05$) using a two-tailed t-test. Similarly, the average internalization time during the 2-cell stage was 3:20 ± 2 min for WEH142 (n=11), 2 min for WEH260 (n=1), 1 min for WEH271 (n=1) and 4:20 ± 1:30 min for WEH296 (n=3) before the 3-cell stage. Again, all strains were not significantly different from each other ($p>0.05$), allowing us to merge the data for cumulative analysis. In summary, internalization happened either 3:30 ± 1:10 min (n=16) before the 3-cell stage or 6:40 ± 1:20 min (n=51) after the start of the 3-cell stage (see Fig. 10A).

To test whether the 2nd polar body is internalized by a stereotyped cell, we next examined which cell engulfed the polar body. As polar bodies are extruded on the anterior side of the embryo (Fabritius et al. 2011; Olson et al. 2012) and therefore located next to the anterior blastomere (AB) cell, we suspected that the 2nd polar body is internalized by the AB-cell or one of its descendants ABa or ABp. For this analysis, we had additional time-lapse data provided by Zhirong Bao, which was annotated by Jaime Lisack and is used for my cumulative analysis. If internalization happened at the 2-cell stage, the polar body was taken up by the anterior blastomere, the AB cell (n=23/24, Fig 10B). If the polar body was not taken up during the 2-cell stage, it ended up between the two daughters of the anterior blastomere when the AB cell divided. When the 2nd

polar body was internalized at the 4-cell stage, it was taken up by either one of the two anterior blastomeres, by ABa (n=27/61) or ABp (n=33/61; Fig. 10B). The remaining four polar bodies were internalized by P1, EMS, MS, and ABaxx, indicating that other cells are also capable of phagocytosis. Thus, the 2nd polar body is internalized by its neighboring cell, which due to its anterior position is normally the AB or ABa/ABp-cell.

In conclusion, the 2nd polar body is internalized in a highly stereotyped manner with internalization either happening during the 2-cell or 4-cell stage. Internalization by the AB cell did not occur during its division, suggesting cytokinesis blocks phagocytosis of the 2nd polar body.

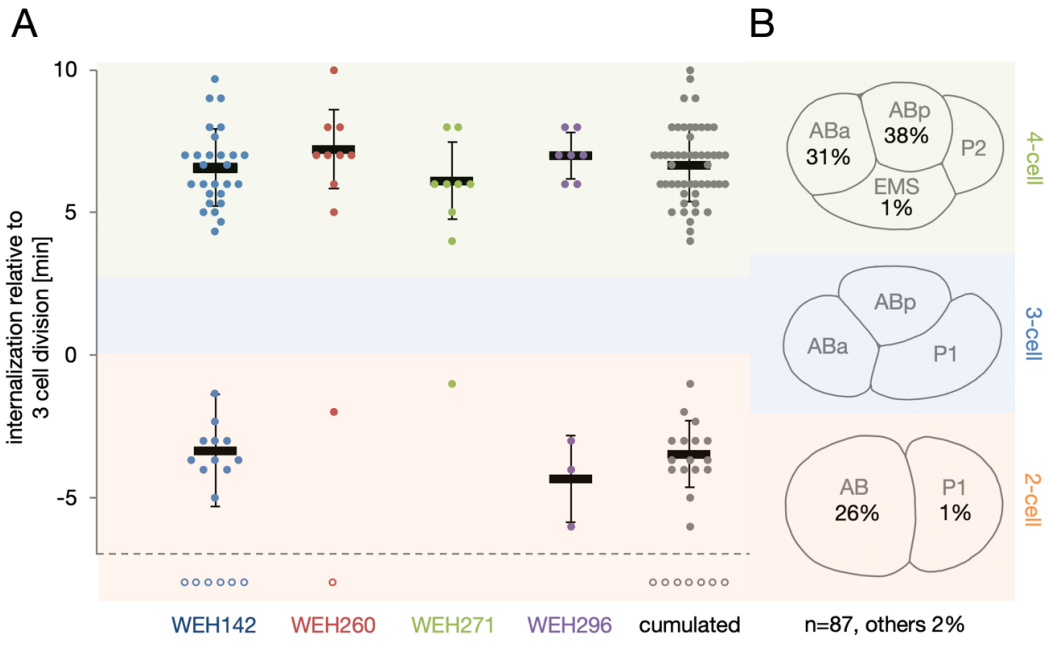


Figure 10 – Stereotyped internalization of the 2nd polar body.
(A) The 2nd polar body is internalized either during the 2-cell or 4-cell stage. Each dot represents one polar body being internalized relative to division of the AB cell (start of the 3-cell stage). Circles below the dashed line represent polar bodies which were already internalized at the beginning of the time lapse, therefore likely to be internalized during the 2-cell stage, but with unknown time point. All worm strains do not show a significant difference in internalization time and can therefore be cumulated (grey dots). The colored background marks the cell stage at each time point (orange=2-cell stage, blue=3-cell stage, green=4-cell stage). There were no internalizations during the 3-cell stage. *The bars show the average internalization time and standard deviation (SD) during either the 2- or 4-cell stage.*
(B) Schematic drawings show the embryo at 2-, 3- and 4-cell stage. Cells names are labeled, and the percentage indicates the proportion of polar bodies which are internalized by this cell. The remaining 2% are not depicted as they were internalized in later cell stages. Total numbers: AB=23, P1=1, ABa=27, ABp=33, EMS=1, MS=1, ABaxx=1

3.3.3 The 2nd polar body is internalized via CED-10/Rac-dependent phagocytosis

Since actin accumulates around the 2nd polar body before and until internalization (Fazeli et al. 2018), we wanted to score the timing of it to get a perspective of actin involvement in the internalization process. Using a LifeAct actin reporter, I scored the onset of actin filament accumulation around the 2nd polar body (see Fig. 11A), revealing that actin starts accumulation up to 4 minutes before the internalization of the 2nd polar body and dissolves quickly after internalization. This data further supports that actin forms the phagocytic cup for polar body internalization and we propose that actin localization could explain the blockage of internalization during cell division.

Actin rearrangement is known to be caused by a few internalization factors, including CED-1 and CED-2. G. Fazeli in our lab also showed that the internalization of the 2nd polar body is driven by receptor-mediated phagocytosis, depending on cell corpse engulfment genes CED-1 and CED-2 (Fazeli et al. 2018). As the CED-2 signaling pathway activates the Rac1 ortholog CED-10, I asked whether internalization was dependent on CED-10. To examine this, I depleted CED-10 by RNAi in a worm strain expressing fluorescent PH marker to label the plasma membrane and a histone marker in nuclei and polar bodies. I took pictures from the 6- to 47-cell stage to see if the polar body was internalized. Out of 21 embryos, 14 did not internalize the polar body (see Fig. 11B, D). Compared to control embryos (Fig. 11B-C), where 98% internalize, internalization rates are significantly lowered ($p<10^{-9}$). Thus the 2nd polar body is internalized via CED-10/Rac-dependent phagocytosis.

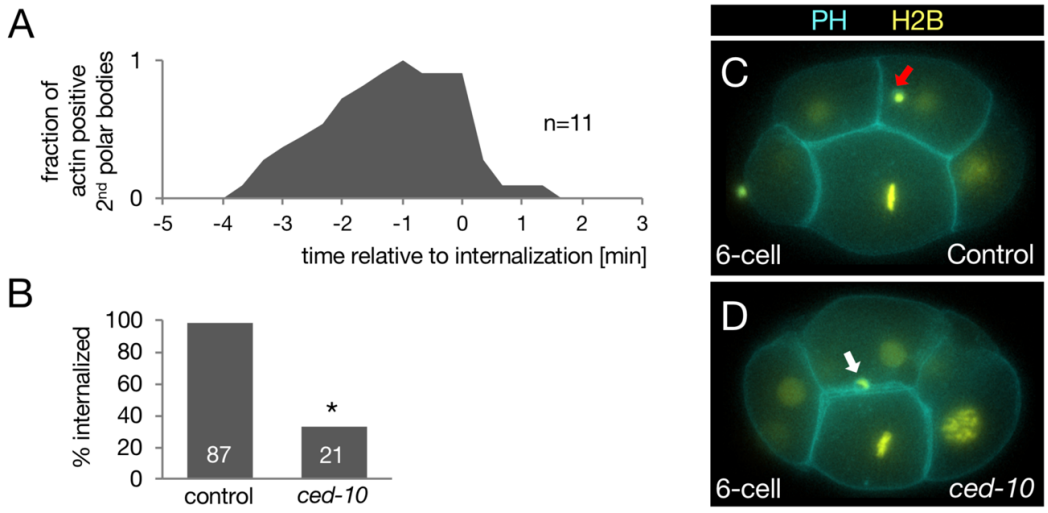


Figure 11 – The 2nd polar body is internalized via CED-10/Rac-dependent phagocytosis.
(A) Timing of actin accumulation around the 2nd polar body. Actin starts accumulating a few minutes prior to polar body internalization and dissolves quickly after internalization.
(B) When depleted of CED-10 by RNAi, 14 out of 21 embryos did not internalize the 2nd polar body. *White numbers indicate total n, *p<10^{-9} using Fisher's exact test.*
(C) At the 6-cell stage, the 2nd polar body (red arrow) is internalized.
(D) When depleted of CED-10, the 2nd polar body (white arrow) is not internalized by the embryo by the 6-cell stage.
Panels C and D were published in Fazeli, G. et al., CellReports, 2018.

3.3.4 SNX-6 regulates phagocytosis via CED-1 trafficking

G. Fazeli observed that the internalization of the 2nd polar body is dependent on the receptor CED-1. To test whether CED-1 is trafficked to the 2nd polar body before internalization, G. Fazeli stained embryos with CED-1 antibody and took pictures, which I scored for CED-1 colocalization with the polar body. CED-1 puncta accumulate around the 2nd polar body before internalization and persist around the phagosome soon after internalization (see Fig. 12A-C), suggesting that CED-1 is recruited to the 2nd polar body to signal for its internalization. CED-1 is rarely observed on the polar body phagosome during the 6- to 8-cell stage, suggesting that it is recycled from the phagosome membrane within 10 minutes after internalization. Thus, CED-1 receptor is actively trafficked to and from the polar body.

The sorting nexin SNX-6 is a retromer-associated protein required for the trafficking of CED-1 (Chen et al. 2010). Therefore, we hypothesized that CED-1

is trafficked by SNX-6 to the 2nd polar body. To test for that, I analyzed the accumulation of CED-1 around the 2nd polar body in *snx-6* RNAi treated embryos. Out of 15 embryos, not a single one showed localization of CED-1 around the 2nd polar body (see Fig. 12D-E), indicating that CED-1 trafficking to the 2nd polar body depends on SNX-6.

We next asked whether polar body internalization is blocked when SNX-6 is missing. To test for that, I depleted embryos of SNX-6 by RNAi and took pictures between the 6- to 12-cell stage. Internalization was significantly blocked after *snx-6* RNAi (see Fig. 12F). Thus, SNX-6 is required for polar body internalization, probably via trafficking of CED-1 to the 2nd polar body.

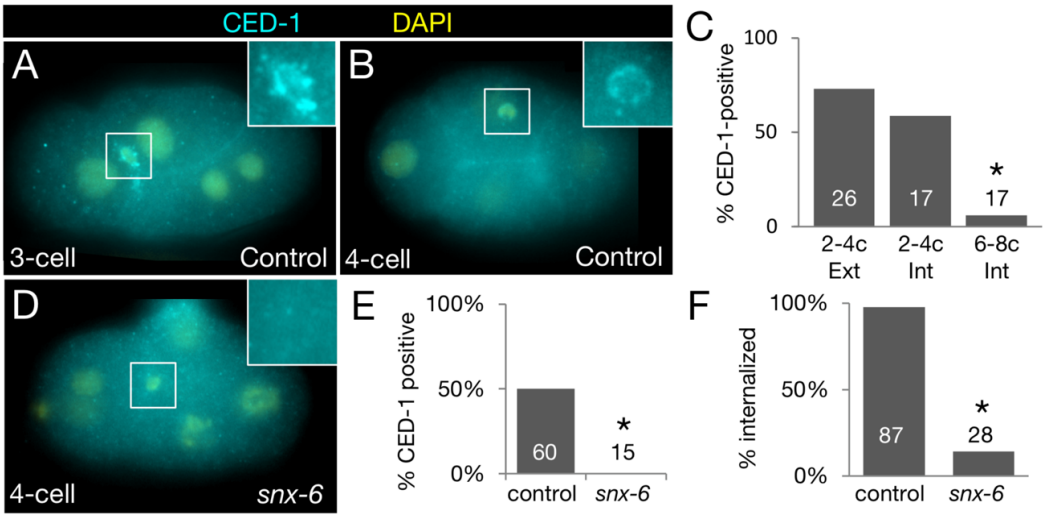


Figure 12 – SNX-6 regulates phagocytosis via CED-1 trafficking.
(A) Shortly before internalization, CED-1 puncta accumulate on the 2nd polar body (white square) in a 3-cell stage embryo.
(B) CED-1 localizes to the polar body phagosome after internalization during the 4-cell stage.
(C) Quantification of CED-1 positive 2nd polar bodies before (Ext) and after internalization (Int). CED-1 is lost within ~10 minutes after internalization in 6-8-cell stage (*p<0,0005 using Fisher's exact test).
(D) In embryos depleted of SNX-6 by RNAi, there are no CED-1 puncta found on the non-internalized 2nd polar body.
(E) Quantification of CED-1 positive 2nd polar bodies in control embryos and embryos depleted of SNX-6 (*p<0,0005 using Fisher's exact test).
(F) Quantification of internalization rates of control embryos and SNX-6 depleted embryos (*p<0,0005 using Fisher's exact test).
Numbers on bars indicate n. Panel A, B and D were published in Fazeli, G. et al., CellReports, 2018.

3.3.5 SNX-1 accumulates on the polar body phagosome

As SNX-6 is needed for the internalization of the 2nd polar body, we next asked if its heterodimer partner SNX-1 is also found on the 2nd polar body during the internalization process. SNX-1 is involved in retromer trafficking of transmembrane receptors like CED-1 (Chen et al. 2010), but also plays a role in phagosome maturation (Lu et al. 2011). To see whether SNX-1 accumulates around the 2nd polar body, time lapse movies of a strain expressing a fluorescent GFP::SNX-1 reporter and a mCherry::H2B polar body marker were made.

GFP::SNX-1 colocalized as punctae with the 2nd polar body starting 1 ± 1 minute (n=10) before internalization and lasting on the polar body phagosome for nearly 4 min (3:40 ± 1:20 min; see Fig. 13B). After internalization, GFP::SNX-1 formed a ring around the phagosome (see Fig 13A). Thus, as SNX-1 accumulated on the polar body shortly before and after internalization SNX-1 is likely to be involved in the internalization of the 2nd polar body along with SNX-6. The quick dissociation of GFP::SNX-1 from the polar body phagosome is similar to CED-1, which is normally dissociated from the phagosome by the 6-cell stage (6:30 minutes after internalization, assuming internalization happened during the 4-cell stage; see section 3.3.4). These results support the involvement of SNX-1 and SNX-6 in recycling proteins like CED-1 from the maturing polar body phagosome back to the plasma membrane within minutes of phagocytosis.

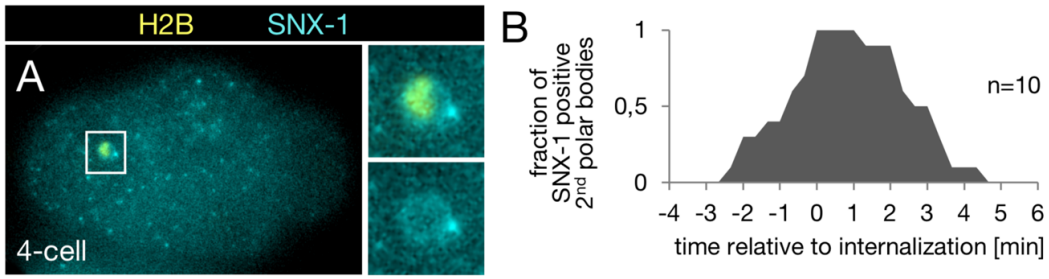


Figure 13 – SNX-1 accumulates on the 2nd polar body.
(A) Shortly after internalization, GFP::SNX-1 starts forming a ring around the 2nd polar body. Embryo expressed mCherry::H2B to label chromosomes of the polar body and GFP::SNX-1.
(B) Timing of SNX-1 accumulation on the 2nd polar body, showing the fraction of GFP::SNX-1-positive polar bodies for each time point.

3.3.6 The 2nd polar body rides the AB ingression furrow, ending up between the newly formed ABa- and ABp-cell

In most embryos, internalization happens during the 4-cell stage and the 2nd polar body shows a stereotyped movement before its internalization. After extrusion, the polar body is moved around on the anterior side of the embryo as the embryo rotates, but it does not leave its anterior position. During the AB cell division, the 2nd polar body is typically dragged by the AB ingression furrow in between the newly formed ABa and ABp cells (77%, n=13/17, see Fig. 5A). This interblastomeric movement of the 2nd polar body suggests that adhesive contacts are formed between the 2nd polar body and its neighboring cell, which pull the 2nd polar body into the ingression furrow. To find possible factors that could bind to the 2nd polar body and drag it during the AB cell division, I analyzed movies with various gene depletions that alter cell-cell adhesion or internalization.

3.3.6.1 RAB-5 and VPS-34 regulate the movement of the 2nd polar body into the AB-ingression furrow

As CED-1 receptor accumulates around the 2nd polar body before its internalization (see section 3.3.4), we hypothesized that CED-1 or other engulfment pathways could also play a role in the interblastomeric movement. However, the polar body still performed its interblastomeric movement during the AB cell division in CED-1-depleted embryos (73%, n=8/11; see Fig. 14). Thus, the CED-1 receptor does not adhere to the polar body and is not necessary to perform the interblastomeric movement. Similar results were seen when embryos were depleted of other factors involved in phagocytosis and actin polymerization, including CED-2 (68%, n=15/22) and CED-10 (80%, n=4/5). This suggests that the classical engulfment pathways do not play a role in interblastomeric movement (see Fig. 14).

We next examined proteins involved in vesicular trafficking, which may alter the localization of many plasma membrane proteins, such as RAB-5 GTPase and the PI3Kinase VPS-34. If either of those proteins is depleted by RNAi, the interblastomeric movement of the 2nd polar body happens significantly

less often (*rab-5*: 20%, n=4/20, p=0.0031; *vps-34*: 21%, n=3/14, p=0.007; see Fig. 14), suggesting both proteins affect the movement of the 2nd polar body prior to internalization.

To test the idea that the cell and polar body are adhering, we next wanted to disrupt cell-cell adhesion in the embryo. We suspected that adhesion of the 2nd polar body could be disrupted if the embryo was depleted of TAT-5, a flippase that inhibits the formation of extracellular vesicles (EVs) (Wehman et al. 2011). EVs accumulate if TAT-5 is depleted, which results in poorly adhering, rounded cells. Interblastomeric movement of the 2nd polar body occurred less often after TAT-5 depletion (n=5/10), however this result was not significantly different when compared with controls (p=0.22). Thus, a higher n is needed to test whether TAT-5 depletion disrupts contacts between the AB cell and the 2nd polar body.

In summary, we hypothesize that physical forces from the AB cell division are pulling the 2nd polar body into the ingression furrow using adhesive contacts between the polar body and the AB-cells. RAB-5 and VPS-34 likely play a role in regulating the interblastomeric movement of the 2nd polar body through their role in membrane trafficking and further experiments are required to identify the specific adhesion factors involved.

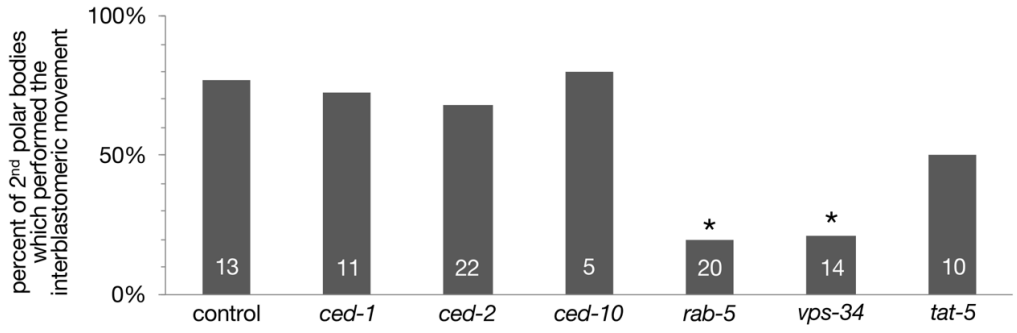


Figure 14 – RAB-5 and VPS-34 are required for the interblastomeric movement of the 2nd polar body. Bars showing the fraction of polar bodies which performed the stereotyped interblastomeric movement of the 2nd polar body. *Numbers on bars indicate n.* (control n=10/13; *ced-1* n=8/11, p=1; *ced-2* n=15/22, p=0.71; *ced-10* n=4/5, p=1; *rab-5* n=4/20, p=0.0031; *vps-34* n=3/14, p=0.007; *tat-5* n=5/10, p=0.22; p-values were calculated using Fisher's exact test.)

3.4 The maturation of the polar body phagosome

The clearance of phagocytosed contents is important to avoid an autoimmune response in mammals. If a cell like a macrophage is able to engulf dead cells, but lacks clearance function, it produces inflammatory cytokines, which can manifest as lupus-like disease in mice (Martinez et al. 2016). Therefore, studying clearance mechanisms after engulfment is essential to understand the origin of autoimmune disease.

Polar bodies are the first cell corpses that the embryo interacts with during embryonic development. As the 2nd polar body carries chromosomes that we can tag with fluorescent reporters, we can easily track the 2nd polar body throughout the process of maturation. Given the large size difference between the embryonic nuclei and polar bodies, as well as their stereotyped pattern of internalization, the second polar body presents a robust new model system to study the mechanisms of cell corpse clearance as well as phagosome maturation and resolution.

After the internalization of the 2nd polar body, we found that it takes over two hours until the fluorescence of polar body reporters is lost. In this time period, we discovered that the polar body phagosome matures in a highly choreographed process. We investigated this process using time-lapse imaging to gain insight into *in vivo* accumulation and dissociation of various maturation factors. Learning about the clearance of the 2nd polar body in *C. elegans* can help us better understand cell corpse clearance and the origins of autoimmune disease in mammals.

3.4.1 The polar body phagosome acidifies and degrades its content

Phagosomes mature and degrade their content by gradually acidifying, aiding the denaturation of their protein content (Flannagan et al. 2012). To characterize the timing of phagosome acidification, we made time lapse movies using mCherry- and GFP-tagged histone reporters. These proteins have different properties in terms of fluorescence intensity at different pH-levels. GFP loses fluorescence already at slightly acidic pH values (pKa~6) (Kneen et al. 1998),

whereas mCherry loses its fluorescence only at lower pH-values (pKa<4.5) (R. A. Green et al. 2008). Therefore, mCherry is expected to fluoresce longer than GFP during the continuous acidification process of the phagosome.

I made movies of a worm strain expressing GFP-labeled histones and analyzed these movies for the last time point the 2nd polar body was trackable. I also analyzed movies made by Jaime Lisack of a worm strain expressing mCherry-labeled histones and compared these data. GFP fluorescence disappeared from the 2nd polar body 40 ± 15 min after internalization (n=16), whereas mCherry fluorescence persisted 87 ± 23 min (n=11) after internalization (Fig. 15B), consistent with phagosome acidification. To confirm this directly, I crossed the GFP- and mCherry reporters together and observed their fluorescence. Consistent with the two individual strains, the strain expressing both markers lost GFP fluorescence in the 2nd polar body long before pH-stable mCherry (Fig. 15A), whereas embryonic nuclei remained labeled with both GFP and mCherry. Thus, the phagosome gradually acidifies before degrading its contents.

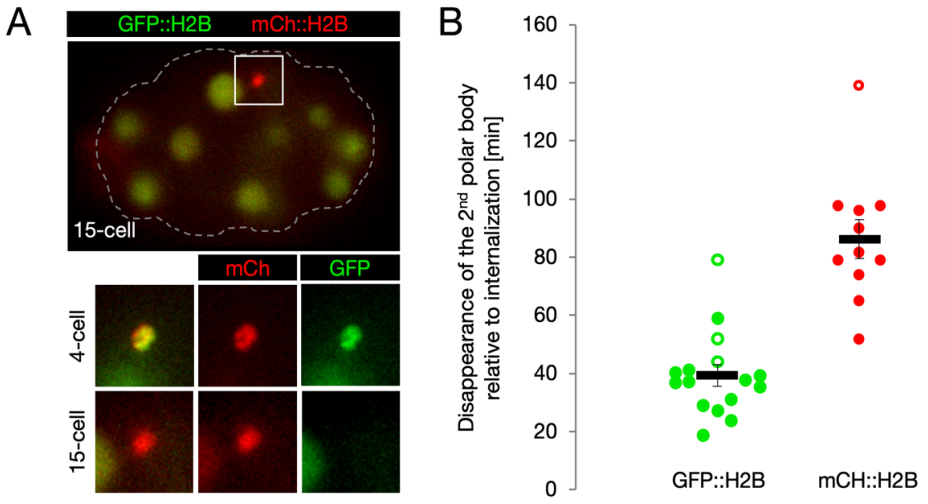


Figure 15 – The polar body phagosome gradually acidifies.
(A) 4-cell stage embryo expressing fluorescent GFP::H2B and mCherry::H2B marker shows both green and red fluorescence in the 2nd polar body (small boxes). By the 15 cell-stage, the fluorescence of GFP is lost, as the polar body phagosome has acidified. Embryonic nuclei are still fluorescent for both markers at the 15-cell stage, as they do not acidify.
(B) The 2nd polar body is observed significantly later in mCherry-tagged H2B embryos than in GFP-tagged H2B embryos. *Open circles represent embryos where the polar body had not yet disappeared at the point when the movie ended; in these cases, the last time point of the movie is shown in the graph. Bars represent mean and standard error of the mean (SEM).*

3.4.2 The polar body phagosome acquires the Rab GTPases RAB-5 and RAB-7

Rab GTPases are a family of proteins which are localized on the cytosolic side of specific intracellular membranes. They function as regulators in membrane trafficking pathways, helping endosomes to mature (Stenmark & Olkkonen 2001). For example, RAB-5 is involved in the formation of early endosomes and indirectly recruits RAB-7 (Fairn & Grinstein 2012), while RAB-7 marks late endosomes and is required for lysosome tethering and fusion (Harrison et al. 2003). We asked whether these GTPase proteins are also involved in the maturation of the polar body phagosome.

I crossed GFP-tagged reporters for RAB-5 and RAB-7 with a polar body marker and made time lapse movies to characterize the timing of phagosome maturation. RAB-5 colocalized with the polar body phagosome within 20 ± 20 seconds after internalization (n=9) and disappeared on average 60 ± 20 seconds later. RAB-7 appeared on the polar body phagosome 80 seconds after internalization (± 100 sec) and persisted for 16 ± 4 min (n=10). Therefore, the polar body phagosome matures by briefly acquiring the Rab GTPase RAB-5, which is rapidly exchanged for RAB-7.

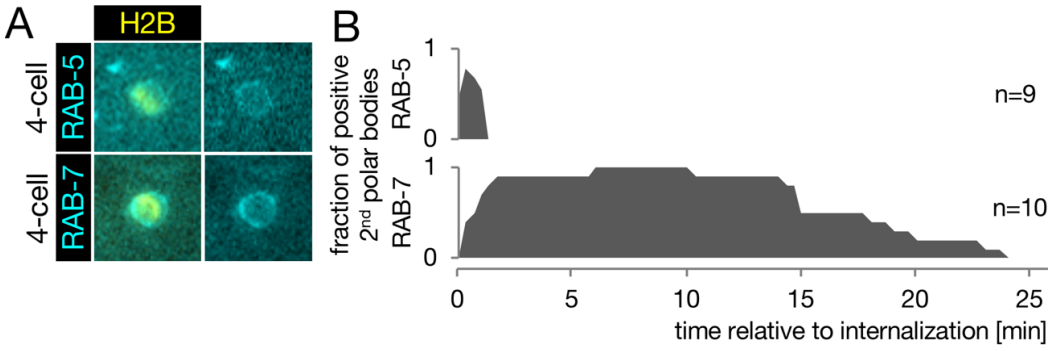


Figure 16 – The polar body phagosome acquires and turns over the Rab GTPases RAB-5 and RAB-7.
(A) GFP::RAB-5 and GFP::RAB-7 both form a ring around the 2nd polar body shortly after internalization. Embryos express a fluorescent H2B marker to label histones and fluorescent RAB-5 or RAB-7 reporters.
(B) Timing of GFP::RAB-5 and GFP::RAB-7 recruitment showing the fraction of positive polar bodies relative to internalization.

3.4.3 Lysosomes are recruited to the 2nd polar body phagosome shortly after internalization

Phagosome maturation is characterized by fusion of the phagosome with lysosomes to become a phagolysosome (see Fig. 1). Therefore, we expected the polar body phagosome to become positive for the lysosome-associated membrane protein (LAMP) homolog LMP-1. To confirm this, I first analyzed whether I could detect LMP-1 positive 2nd polar bodies from existing stainings with LMP-1 antibody provided by Katharina Beer from our lab. I was able to identify 40 2nd polar bodies in embryos between the 2- and 15-cell stage, from which 23 were positive for LMP-1 puncta (see Fig. 17A). Thus, the 2nd polar body phagosome becomes positive for LMP-1, indicating that lysosomes are recruited to the polar body phagosome.

I next asked when the polar body phagosome starts recruiting lysosomes and how long they persist on it. We hypothesized that lysosomes appear on the phagosome shortly after RAB-7 recruitment, as RAB-7 recruits lysosomes (Harrison et al. 2003). I crossed a LMP-1::GFP reporter with a polar body marker and recorded time-lapse movies. In contrast to the LMP-1 antibody staining, the LMP-1::GFP reporter appeared weakly on the plasma membrane of embryonic cells in addition to intracellular puncta, which may be due to overexpression of the multicopy transgene or the position of the GFP tag. Therefore, the polar body phagosome membrane was weakly LMP-1-positive directly after internalization, as the phagosome membrane is derived from the LMP-1::GFP-positive plasma membrane (see Fig. 17B).

To score for the timing of lysosome recruitment after internalization, I looked for the time point when the polar body phagosome showed an increase in LMP-1::GFP fluorescence over the plasma membrane (see Fig. 17C). Using this criteria, LMP-1::GFP appeared on the polar body phagosome 6 ± 5 min after internalization (Fig. 17D, n=10). Thus, lysosomes were recruited around 5 minutes after the 2nd polar body phagosome became positive for RAB-7 (see section 3.4.2), consistent with a conserved role of RAB-7 as a lysosome recruit-

ment factor. I also observed that LMP-1::GFP persisted until the phagolysosomal cargo disappeared (Fig. 17D), in contrast to the turnover of RAB-7 (Fig. 17B). Thus, the polar body phagosome recruits lysosomes shortly after internalization, which persist on the phagolysosome through degradation, consistent with a role for lysosomal hydrolases in degradation of the polar body.

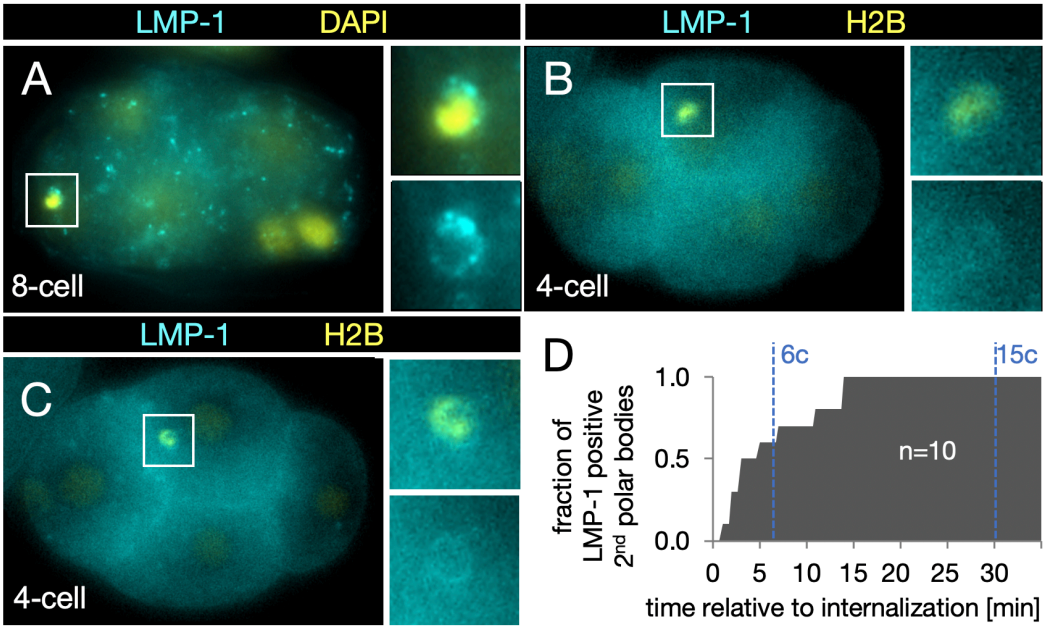


Figure 17 – Lysosomes are recruited to the 2nd polar body phagosome shortly after internalization.
(A) LMP-1 antibody stains a ring around the 2nd polar body in an 8-cell embryo.
(B) Directly after internalization, LMP-1::GFP can be found forming a weak ring around the 2nd polar body. However, since its fluorescence is not stronger than the plasma membrane, this was not scored as a positive recruitment, as its fluorescence likely derives from the plasma membrane.
(C) Later after internalization, LMP-1::GFP is enriched on the 2nd polar body phagosome, clearly brighter than the plasma membrane.
(D) Timing of lysosome recruitment to the polar body phagosome. The polar body phagosome remains positive for LMP-1::GFP until its degradation. *6-cell and 15-cell stage are labeled in blue for orientation, assuming internalization happened during the 4-cell stage*.

3.4.3.1 RAB-7 dependent lysosome recruitment aids the degradation of the 2nd polar body

We next tested whether RAB-7 is needed for lysosome recruitment to the polar body phagosome. I first depleted RAB-7 by RNAi and took pictures of LMP-1::GFP embryos between the 6- and 12-cell stages. From 20 embryos, only 6 polar body phagosomes colocalized with LMP-1, which is significantly lower

compared to the time lapse control data (p=0.0003), which were all positive for LMP-1 during these stages (see Fig. 18A).

We next examined the timing of lysosome recruitment in time lapse movies of RAB-7-depleted embryos taken for 30 minutes after internalization. Out of 10 *rab-7-RNAi* treated embryos, eight showed no visible recruitment to the polar body phagosome within 30 minutes of internalization (Fig. 18B-C). In the two *rab-7*-depleted embryos with recruitment of LMP-1 to the polar body phagosome, colocalization occurred 22 minutes after internalization (Fig. 18C). This is in contrast to wild type, where LMP-1 recruitment occurs after 6 minutes (Fig. 82C). Thus, LMP-1 recruitment is significantly delayed compared to controls when RAB-7 is depleted (p=0.0003), confirming that RAB-7 plays an important role in lysosome recruitment to the polar body phagosome.

As lysosome fusion aids the degradation of phagocytosed cargo, we expected polar bodies to persist longer in embryos that are defective in lysosome fusion due to RAB-7 depletion. To test this, I analyzed the degradation time in *rab-7* RNAi-treated embryos expressing a mCh::PH::ZF1 marker to label the polar body's membrane. On average, polar body reporters lost fluorescence significantly later after *rab-7* depletion: 142 ± 12 min (n=11, p<0.001; see Fig. 18D), which is almost an hour later than controls. Therefore, RAB-7-dependent lysosomal recruitment to the phagosome aids the degradation of the 2nd polar body.

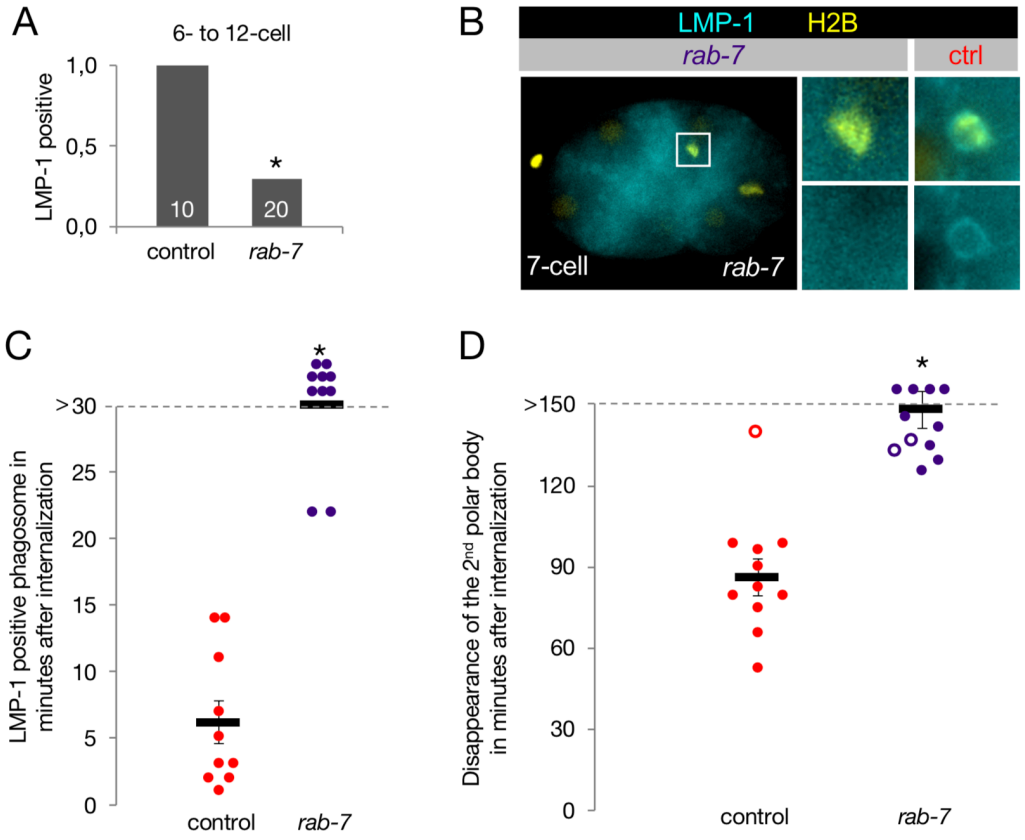


Figure 18 – Lysosome recruitment to the phagosome depends on RAB-7 and aids the degradation of the 2nd polar body.
(A) When depleted of RAB-7, there is a significantly lower colocalization rate of LMP-1::GFP in 6- to 12-cell stage embryos compared to controls (*p=0.0003).
(B) 15 min after internalization during the 7-cell stage in RAB-7-depleted embryos, there is still no visible colocalization of LMP-1::GFP with the polar body phagosome, while control embryos (boxes on the right side, 4-cell stage) show a LMP-1::GFP ring around the polar body.
(C) Timing of LMP-1 recruitment in control untreated embryos and after *rab-7* RNAi treatment shows a significant delay of LMP-1 recruitment in RAB-7-depleted embryos (*p=0.0003 using Student's t-test). Data points above the dashed line indicate embryos with no LMP-1 recruitment within 30 minutes of internalization.
(D) Timing of polar body disappearance in embryos expressing mCh::PH::ZF1 reveals that polar bodies persist significantly longer in embryos treated with *rab-7* RNAi (*p<0.0001 using Student's t-test).
Points show last frame in which the 2nd polar body was visible; open circles indicate the last frame of the time lapse in which the 2nd polar body persisted until the end of imaging. Data points above the dashed line indicate polar bodies which persisted for more than 150 minutes. Bars show average and standard error of the mean (SEM).

3.4.4 The role of LC3-associated phagocytosis in the maturation of the 2nd polar body phagosome

Membrane-wrapped cell debris, such as embryonic midbody remnants, are internalized and degraded via LC3-associated phagocytosis (LAP, see Fig.

1B) in *C. elegans* embryos (Fazeli & Wehman 2017a). We hypothesized that this could also apply for the 2nd polar body, as it is a membrane-wrapped cell corpse. To test for that, I analyzed whether factors involved in LAP colocalize with the polar body phagosome. As many factors involved in LAP are also involved in autophagy, including LC3, I tested whether LC3 colocalization with the polar body could be due to increased autophagy within the 2nd polar body and whether phagocytosis is needed for LC3 recruitment. As LC3 is discussed to play a role in lysosome recruitment (Ktistakis & Tooze 2016), I also tested whether it plays a role there.

3.4.4.1 The 2nd polar body phagosome recruits LGG-1 and LGG-2

We first asked whether factors from LC3-associated phagocytosis (LAP) are involved in the processing of the 2nd polar body. During LAP, proteins from the Atg8/LC3 family are recruited to the phagosome surface. To test whether this is the case in polar body corpse clearance, I examined the localization of the *C. elegans* Atg8/LC3 family proteins LGG-1 and LGG-2. I crossed strains expressing fluorescent GFP::LGG-1 or mCherry::LGG-2 with worm strains expressing mCh::H2B or GFP::H2B to label polar body DNA in order to create double-labeled strains. I made photos of these strains to test whether Atg8/LC3 proteins accumulated on the polar body phagosome. 18 out of 21 embryos showed colocalization of LGG-1 with the 2nd polar body phagosome between the 4- and 7-cell stage (Fig. 19A). 18 out of 29 embryos showed colocalization of LGG-2 with the polar body phagosome in embryos photographed between the 6- and 15-cell stage. There were no LGG-2-positive 2nd polar body phagosomes during the 4-cell stage (n=0/5), suggesting that LGG-1 could be recruited earlier than LGG-2. Alternatively, the LGG-2 reporter could be less robust than the LGG-1 reporter, consistent with our observed lower colocalization rates (86% for LGG-1 vs. 62% for LGG-2). In conclusion, both LC3 homologs are recruited during early stages of polar body phagosome maturation.

I next made time-lapse movies of the LGG-2 reporter strain to better characterize the dynamics of colocalization. LGG-2 appeared on the polar body phagosome 6 ± 6 min (n=13/14) after internalization. In one of the 14 embryos,

the polar body phagosome did not become positive for LGG-2 during imaging, while one was positive for LGG-2 13 minutes before its internalization, possibly due to intrinsic LGG-2 activity. LGG-2 appeared on average simultaneously with LMP-1, which could suggest a similar role for LC3 in lysosome recruitment. LGG-2 persisted on the polar body phagosome for 42 ± 17 minutes before disappearing like the Rab GTPases (Fig. 19B). The removal of LGG-2 before the degradation of the phagolysosome cargo is similar to LC3 removal in autophagy before autolysosome degradation (Kirisako et al. 2000). Thus, LGG-2 localizes to the polar body phagosome during its maturation and could play a role in polar body clearance. However, in contrast to LMP-1, RAB-5 and RAB-7, which form a ring on the surface of the phagosome, LGG-1 and LGG-2 appeared to fill the phagosome (see Fig. 19A). This raised the possibility of intrinsic LGG-1/-2 activity in the 2nd polar body, perhaps due to increased autophagy within the dying cell (Nikoletopoulou et al. 2013).

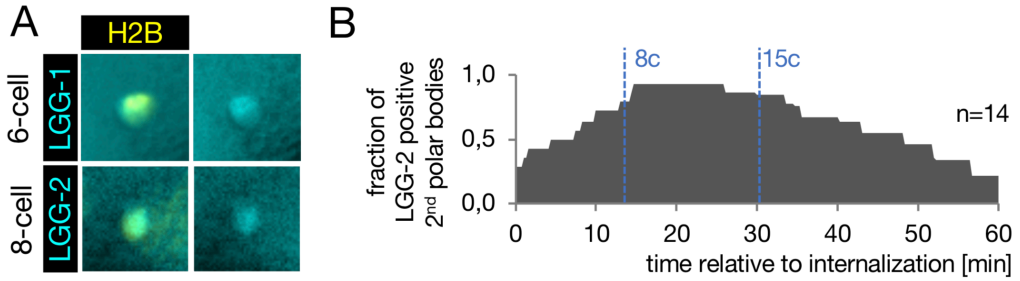


Figure 19 – LGG-1 and LGG-2 accumulate in the polar body phagosome.
(A) GFP::LGG-1 and LGG-2::mCh colocalize with the 2nd polar body phagosome after its internalization. Embryos are expressing fluorescent LGG-1 or LGG-2 marker (cyan) and a histone marker (yellow) to label the polar body.
(B) Timing of LGG-2::mCh recruitment to the 2nd polar body phagosome relative to internalization. *The beginning of the 8- and 15-cell stage are indicated in blue for orientation, assuming internalization happened during the 4-cell stage.*

3.4.4.2 LGG-2 accumulation on the 2nd polar body depends on phagocytosis, not on macroautophagy-specific EPG-8

As dying cells sometimes increase autophagy (Nikoletopoulou et al. 2013) and we observed LGG-1 and -2 colocalization with the phagolysosome content as well as one 2nd polar body being positive for LGG-2 before its internalization,

we hypothesized that LGG-1/2 colocalization could be due to increased autophagy within the 2nd polar body and be independent of phagocytosis. To test these hypotheses, we depleted CED-1 to prevent internalization and examined whether LGG-2 still accumulated in the 2nd polar body without phagocytosis. I took photos of these embryos between the 8- and 15-cell stage, as colocalization rates were the highest at these time points (see Fig. 19B) Out of 15 non-internalized 2nd polar bodies photographed between the 8- and 15-cell stage, only 3 showed visibly enriched LGG-2 fluorescence (13%), significantly less than in untreated controls, where 14 of 19 embryos (74%) showed colocalization between the 8- and 15-cell stage (p=0.0006, see Fig. 20A). Therefore, colocalization of LGG-2 with the polar body is largely dependent on phagocytosis and not only due to increased autophagy within the dying polar body.

As LC3 could also accumulate due to autophagosome fusion with the phagosome (Amer & Swanson 2005), I next tested whether LC3 recruitment to the phagosome required macroautophagy by examining an *epg-8* null mutant strain. EPG-8 is a macroautophagy-specific component of the Class III PI3Kinase complex (P. Yang & Zhang 2011), which is not required for LAP (Fazeli et al. 2016)(see Fig. 1B-C). I took photos of *epg-8* mutant embryos from the 8- to 15-cell stage, when LGG-2 normally has the highest likelihood to accumulate on the phagosome (Fig. 10B). The colocalization rates in *epg-8* mutant embryos (19/25 positive, 76%) were not significantly different compared to control embryos (14/19 positive, 74%, p=1; see Fig. 20B). Therefore, EPG-8 is not needed for LGG-2 recruitment to the 2nd polar body phagosome, which suggests that LGG-2 localization is not due to autophagy. Taken together, these data suggest that LC3 specifically associates with the polar body phagosome, consistent with LAP.

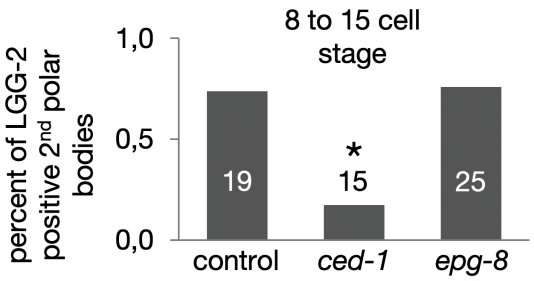


Figure 20 – LGG-2 accumulation on the polar body phagosome depends on phagocytosis, not on macroautophagy.
When depleted of the engulfment receptor CED-1 by RNAi, LGG-2 colocalizes significantly less often with the polar body (*p=0.0006 using Fisher's exact test). In EPG-8 deletion mutant embryos defective for macroautophagy, there is no significant difference in comparison with the control group (p=1 using Fisher's exact test). *Numbers on/above bars represent n.*

3.4.4.3 LC3 is not required for lysosome recruitment to the 2nd polar body LAPosome

LC3 on the autophagosome surface was proposed to play a role in autophagosome-lysosome fusion (Ktistakis & Tooze 2016), so we suspected that disrupting LC3 recruitment could cause a failure of lysosome fusion with LC3-associated phagosomes. Therefore, we asked whether the Atg8/LC3 homologs LGG-1 and LGG-2 are recruited to the polar body phagosome to increase lysosome fusion. I treated embryos expressing a polar body marker and the lysosome marker LMP-1 with *atg-7*-RNAi to decrease LGG-1 and LGG-2 recruitment to the phagosome. ATG-7 is needed for lipidation of LGG-1 and LGG-2 and therefore the function of those factors during both macroautophagy and LAP (Tian et al. 2010). I took photos of 6- to 15-cell stage embryos and analyzed them for colocalization of LMP-1 with the polar body, as 100% of polar bodies had recruited LMP-1 by the 6-cell stage in our time-lapse analysis (Fig. 17D). LMP-1 colocalized with the polar body phagosome in all 18 *atg-7* knockdown embryos, suggesting that ATG-7 and therefore LC3 lipidation are not essential for lysosome fusion with the polar body phagosome (Fig. 21A).

As this data was taken from still images, we also considered whether the timing of lysosome fusion was altered after LC3 depletion. I analyzed the timing

of LMP-1 recruitment on the polar body phagosome in time-lapse series from embryos treated with *atg-7* RNAi. For specific colocalization criteria, see section 3.4.3. The average first colocalization time was about 7 ± 5 minutes (n=10; see Fig. 21B), which is not significantly different compared to controls (p=0.43). Ergo, this data does not support a general role for LC3 in the fusion of lysosomes with phagosomes, which leaves the function of LC3 in polar body clearance unanswered at this point.

In conclusion, I showed that Atg8/LC3 proteins are recruited to the 2nd polar body depending on phagocytosis of the dying cell. I also demonstrated that macroautophagy does not influence LC3-recruitment to the polar body phagosome. Thus, we propose that the 2nd polar body is likely to be degraded by LC3-associated phagocytosis.

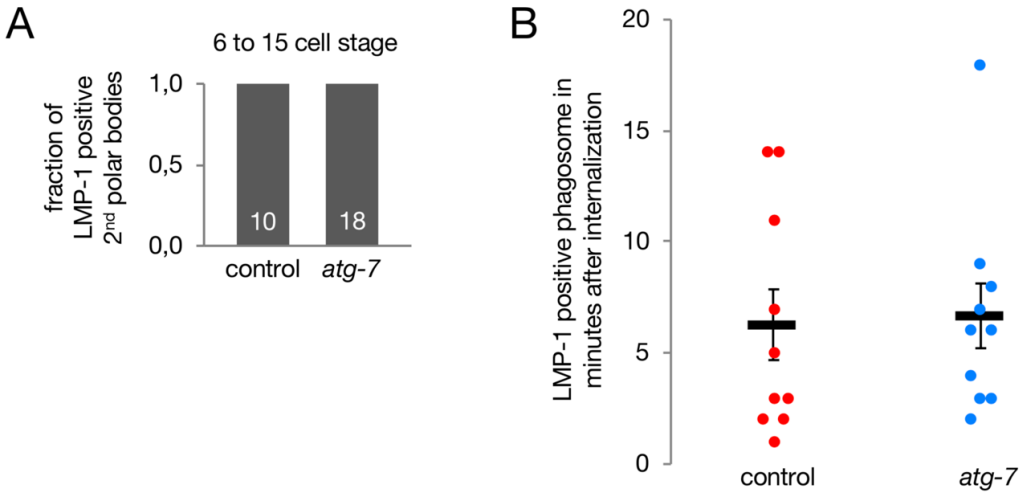


Figure 21 – LC3 is not needed for lysosome recruitment to the 2nd polar body phagosome.
(A) The polar body phagosome still recruited LMP-1::GFP by the 6- to 15- cell stage after depletion of LC3 function by *atg-7* RNAi. Control data was analyzed from time lapse series (see section 3.4.3).
(B) The timing of LMP-1::GFP recruitment was not significantly different from control embryos (p=0.43). Each bar represents mean and standard error of the mean (SEM).

4 DISCUSSION

In order to better understand the mechanisms of cell death and cell corpse clearance, I characterized the death, engulfment and degradation of polar bodies in this work. I revealed proteins involved in these processes to gain a deeper understanding of these fundamental biological processes, which are likely to be conserved in humans. Thus, we established the *C. elegans* 2nd polar body as an attractive biological model for further research on the mechanisms of cell death, phagocytosis, and intracellular degradation, which are important parts of normal physiology as well as disease.

In particular, this work demonstrated that both polar bodies die and lose their membrane integrity, suggesting they undergo a form of programmed necrosis (discussed in section 4.1). The 2nd polar body is in contact with embryonic cells after its extrusion and performs a stereotyped movement before its engulfment (4.2). Engulfment of the 2nd polar body by early embryonic cells depends on engulfment receptors, which need to be trafficked to the plasma membrane (4.3). Degradation of the 2nd polar body takes around 90 minutes and involves the recruitment of various phagosome maturation factors, as well as LC3-associated phagocytosis (4.4). With this research, we have been the first to reveal the function of LC3 in LAP (4.5). Thus, we have established a model system to study cell death, corpse engulfment, phagosome maturation and the role of LC3 during LC3-associated phagocytosis (4.6), which are involved in a variety of processes and associated with disease in humans (4.7).

4.1 Death

The leading cause for polar body death remains unclear. We found that polar body DNA fragmentation and loss of membrane integrity are independent of phagocytosis, as CED-1 depletion still resulted in TUNEL-positive 2nd polar bodies (Fazeli et al. 2018). These data rule out phagoptosis as a potential death mechanism.

We tested whether polar bodies lose membrane integrity, which could lead to the dispersion of cellular contents into the extracellular space. While the 1st polar body was nearly always stained by PI, we had to disrupt the eggshell's

permeability barrier in order to stain the 2nd polar body, as PI was not able to penetrate this barrier. Surprisingly, disrupting the eggshell's permeability barrier by *ptr-2* knockdown resulted in weak staining of both polar bodies. The weak staining could be caused by an incomplete disruption of the permeability barrier of the eggshell. However, the clearly visible staining (see Fig. 7) of the endodermal cells indicates that *ptr-2* knockdown successfully disrupted the permeability barrier of the eggshell. Thus, it appears unlikely that the weak staining is caused by an incomplete disruption of the eggshell.

Alternatively, the weak staining could be due to the mispositioning of the 1st polar body. In addition to weaker PI staining when the 1st polar bodies were mispositioned under the eggshell after *ptr-2* knockdown, we also observed fewer 1st polar bodies staining positive for PI. This raises the possibility that the integrity of the 1st polar body membrane could be further damaged when it is trapped in the eggshell. Using electron microscopy to examine the morphology and positioning of the 1st polar body after *ptr-2* knockdown could provide insight into the cause of these staining differences. In summary, these findings suggest that both polar bodies lose membrane integrity and that differences in PI staining may be due to differences in their positioning relative to the permeability barrier.

Polar bodies also lose membrane integrity within the first hour after their extrusion, indicating that they rapidly undergo a form of necrotic cell death. Since both polar bodies lost membrane integrity shortly after their extrusion, they are not likely to be undergoing secondary necrosis (Elliott & Ravichandran 2016), suggesting a type of programmed cell death leads to an accelerated loss of membrane integrity.

Indeed, polar bodies show signs of programmed cell death but appear not to be apoptotic. Polar bodies were shown to have fragmented DNA using TUNEL staining, even when apoptotic caspase functions are depleted in *C. elegans* (Wu et al. 2000) and mice (Zakeri et al. 2005). We confirmed DNA fragmentation of both polar bodies by TUNEL staining and also found that both polar bodies signal for internalization by externalizing phosphatidylserine (PS) (Fazeli

et al. 2018), a sign of programmed cell death (Conradt et al. 2016). Thus, members of the lab asked whether PS externalization is a result of apoptotic caspase activity. They discovered that the externalization of PS was not dependent on the apoptotic caspase CED-3 or its effector scramblases SCRM-1 and CED-8 (Fazeli et al. 2018; Jörg 2018). As these scramblases are known to work independent of each other and can be regulated by non-apoptotic processes (see section 1.5.1), it should be tested whether double mutants of SCRM-1 and CED-8 show a disruption in PS externalization on polar bodies. Alternatively, the loss of membrane integrity observed with PI staining could also result in PS exposure. Polar bodies are also stained by an antibody which marks engulfed apoptotic cells (Eisenhut et al. 2005). These data suggest that polar bodies undergo a form of non-apoptotic programmed cell death, which shares common steps with apoptosis and displays necrotic features. Whether polar bodies undergo a type of programmed necrosis or other pathways lead to polar body death (Kutscher & Shaham 2017) remains to be determined.

The leading cause for polar body death also remains to be investigated. Polar bodies inherit organelles including mitochondria and endoplasmic reticulum (Dalton & Carroll 2013). Still, energy deprivation could be a cause for polar body death, possibly due to the small size of the polar bodies and lack of nutrients. To test this theory, polar bodies could be removed (similar to a polar body biopsy in humans) and put in a nutrient solution. If deprivation of nutrients was the cause of their death, they should survive under these conditions.

Asymmetric cell division and the subsequent death of the smaller descendant cell is a well-known process during development (Sulston et al. 1983). During embryogenesis, 131 cells are destined to die, and these cells derive from asymmetric cell division, suggesting their fate is decided before they are formed (see section 1.5.1). Thus, it could also be interesting to deplete the LIN-3/EGF-pathway, which has been shown to significantly contribute to cell death of asymmetrically dividing cells during embryogenesis (Jiang & Wu 2014) and analyze whether they contribute to polar body death as well.

Interestingly, in mice, forcing polar body extrusion to be symmetrical led the 2nd polar body to be a fertilizable cell (Otsuki et al. 2012), suggesting the asymmetry not only marks the cell destined to die, but the involved pathways also contribute to its death. Whether that would also apply for the *C. elegans* polar bodies could be tested by depleting MEI-1 or MEI-2 function, which are needed for the asymmetric assembly of the meiotic spindle (Srayko et al. 2000). Their depletion has been shown to produce larger polar bodies, which then could be stained using TUNEL to test whether they die as well.

The polar bodies are the first two cells to die during embryonic development in *C. elegans* and mammals, but it is debatable whether they represent a form of germ cell death or embryonic cell death. As both polar bodies derive from the female germ line of the mother, they could be considered a germ line death. Germ line cell death is a known form of apoptotic cell death which primarily occurs at the turn of the *C. elegans* gonad during oogenesis. This form of cell death is thought to "nurse" other developing oocytes and to select fit oocytes (Gumienny et al. 1999; Huelgas Morales & Greenstein 2018). Just like the germ line cells, polar bodies do not express zygotic genes like *zif-1*, a fact which we used to specifically label them in embryos with ZF1-tagged reporters. Thus, their death could be classified as a non-apoptotic oocyte cell death. However, polar bodies are not extruded until after fertilization in *C. elegans*, making them seem embryonic or extra-embryonic (Dorn et al. 2010). In contrast, the 1st polar body is extruded before ovulation in mammals, while the 2nd polar body is extruded after fertilization (Maro & Verlhac 2002). The 1st polar body can sometimes divide during Meiosis II in mammals, leading to three polar bodies (Schulz-Schaeffer 1980). This suggests that although the 1st polar body is born before fertilization, it may not start dying until after fertilization. Thus, polar body death does not cleanly fit into either category of germ line or embryonic cell death.

One interesting open question is whether the polar bodies are still in meiosis after their extrusion. From their birth, the DNA of the polar bodies remains condensed, which implies that the polar bodies never exit meiosis and enter interphase (Howlett & Bolton 1985). To further test whether polar bodies are still

in a meiotic division program, it might be worth to analyze the expression of *gld-2* and *gld-3*, which are specifically expressed in meiosis, and *glp-1*, which would indicate a mitotic state of the cell (Crittenden et al. 2003). The question remains whether a cell cycle arrest seals the deadly fate of the polar bodies.

In summary, dying polar bodies show signs of both apoptosis and necrosis. Furthermore, their death seems to be tied to the process of meiosis rather than phagocytosis and does not cleanly fit into either category of germ line or embryonic cell death. Therefore, I propose to call this type of programmed cell death “meioptosis”.

4.2 Embryo-Polar Body Contact

Both polar bodies expose signals for internalization, but only the 2nd polar body has contact to embryonic cells and can therefore be engulfed. Adhesive factors are likely to hold the 2nd polar body close to the embryo and allow it to be pulled into the AB-ingression furrow. These adhesive contacts as well as the movement into the AB-ingression furrow likely support polar body engulfment by increasing cell-to-polar-body contact, which would enable maximum binding of eat-me signals to engulfment receptors. Depleting RAB-5 or VPS-34 resulted in significantly fewer polar bodies performing the interblastomeric movement. Both RAB-5 and VPS-34 are known to regulate the trafficking of transmembrane proteins, including CED-1 trafficking (Fazeli et al. 2018). However, CED-1 was not needed for the interblastomeric movement of the 2nd polar body, suggesting that other factors are likely to play a key role. RAB-5 is known to be involved in the recycling of transmembrane proteins, including adhesion proteins like integrins (Bachmann et al. 2019), which could act as an adhesive contact between the polar body and the embryo. Therefore, it is worth investigating whether the integrin-α subunits *ina-1* and *pat-2*, as well as the integrin-β *pat-3* (Rocheleau 2018) are required for the interblastomeric movement of the 2nd polar body.

When embryos were depleted of PTR-2, both the 1st and the 2nd polar body have contact to embryonic cells, which would theoretically enable the internalization of the 1st polar body as well. Indeed, in 3 cases, both polar bodies were internalized, suggesting that 1st polar body successfully presents signals

for engulfment. Therefore, it is likely that the barrier formed by the eggshell prevents the internalization of the 1st polar body. Since the disruption of the eggshell also resulted in fewer engulfed 2nd polar bodies, the eggshell seems to significantly contribute to polar body internalization, possibly due to mechanically increasing the contact between the polar body and embryo. It should be tested whether disruption the formation of the inner layer of the eggshell by depleting *cpg-1/2* (Olson et al. 2012) would lead to a contact of the 1st polar body with the embryo and internalization rates similar to the 2nd polar body, which would suggest that the eggshell is the only reason the 1st polar body is not engulfed.

4.3 Engulfment

Polar body engulfment and clearance was performed by cells neighboring the 2nd polar body on the anterior side, as the polar body gets extruded there. At this early stage in embryonic development, these anterior cells are pluripotent and undifferentiated and can develop into almost any tissue. We have also observed engulfment of the 2nd polar body by the germ line P-cell, however this happens rarely as the 2nd polar body normally has no contact with it due to the posterior position of the cell. In 3 out of 87 cases the polar body was observed to move posterior or was already located at the posterior side next to the P-cell, which enabled engulfment by it. Thus, engulfment of the 2nd polar body is performed by neighboring undifferentiated pluripotent cells and is not restricted to professional phagocytes or to cells of a certain lineage.

We wondered why the embryo risks internalization of the 2nd polar body, as internalization of extra chromosomes could possibly lead to lethal aneuploidy. One hypothesis was that polar body internalization could influence cell fate. Worms lacking engulfment factors are still viable and reproduce, ergo polar body internalization is not essential for the organism but could still contribute to cell differentiation. This could be tested by removing the 2nd polar body and tracking cell differentiation throughout embryogenesis.

Our lab approached this question from a different angle and asked whether the polar body was degraded by a specific cell lineage, suggesting the polar body could influence the cell's fate. They showed that inheritance of the

polar body was random in comparison to the otherwise highly stereotyped embryogenesis (Fazeli et al. 2018). Therefore, polar bodies are not degraded by specific cell lineages, making it unlikely that polar body engulfment and inheritance significantly contributes to cell development. In fact, human polar bodies are routinely removed for genetic testing and so far, there is no study reporting negative effects for the developing embryo and its later life (Desmyttere et al. 2009). However, no randomized-controlled trial has been conducted yet, although it has been recommended by the Preimplantation Genetic Diagnosis International Society (see section 1.4). Thus, there is no evidence to date for a developmental role for polar body engulfment.

Another possible reason for polar body engulfment could be the loss of its membrane integrity. The necrotic polar body is in direct contact with embryonic cells and could induce a stress response of the embryo. Therefore, clearing it could be beneficial for its development. However, engulfment mutants are viable, thus a non-internalized polar body does not pose a lethal danger to the embryo. It could be interesting to test whether factors induced with a pathological stress response increase due to an uncleared 2nd polar body. In mammals uncleared cell corpses can result in the production of auto-antibodies, triggering autoimmune-disease (Martinez et al. 2016). Mammalian polar bodies end up in the zona pellucida, from which the embryo hatches (Gonzales et al. 2001). Hatching may prevent contact of dying polar bodies to the embryo, preventing an embryonic stress reaction. However, there are reports of a persistent contact between the embryo and the 2nd polar body in humans up until after hatching (Ottolini et al. 2015), therefore it should be investigated how often that happens and whether there are any long term negative effects like the development of an autoimmune disease following a prolonged embryo to polar body contact. In conclusion, it remains to be determined why the 2nd polar body is engulfed, although we hypothesize that it is likely to prevent uncontrolled dispersion of its contents due to a loss of membrane integrity.

The 2nd polar body is internalized via receptor-mediated phagocytosis, dependent on the engulfment factors CED-1, CED-2 and CED-10 (see Fig. 2).

Knocking down the engulfment mediator CED-10 significantly lowers internalization rates, although there are other engulfment pathways acting in parallel. This is consistent with results from embryonic corpses, as knocking down one pathway already leads to an increase in persistent cell corpses during embryonic development (Conradt et al. 2016). Consistent with that, internalization rates were also significantly lowered after depleting *ced-1* and *ced-2* (Fazeli et al. 2018).

We have confirmed CED-1 receptor recycling was blocked after SNX-6-depletion and found SNX-1 localizing on the polar body phagosome. Since SNX-1 and SNX-6 act together as a heterodimer, it is likely that both sorting nexins are needed for CED-1 receptor recycling and internalization of the 2nd polar body. This could be tested by depleting *snx-1* and analyzing CED-1 localization as well as internalization rates of the 2nd polar body. These experiments would support our hypothesis that SNX-1 and SNX-6 are recruited to the polar body phagosome together to recycle CED-1 for further rounds of phagocytosis.

4.4 Phagosome maturation

The polar body phagosome matures by acquiring various maturation factors and acidifying. We confirmed that the maturation of the polar body phagosome includes acquiring the Rab GTPases RAB-5 and RAB-7. By analysis of time lapse imaging of embryogenesis, we were able to put the timing of phagosome maturation in perspective. RAB-5 is quickly recruited in under a minute to the polar body phagosome and leaves within another minute. Recruitment and dissociation of RAB-5 and RAB-7 occur faster than reported for cell corpse clearance later in *C. elegans* embryogenesis (He et al. 2010). For example, RAB-7 has been shown to be recruited to over 90% of later cell corpse phagosomes within 10 minutes (Yu et al. 2008; He et al. 2010), while we found that 90% of polar body phagosomes were positive for RAB-7 within 2 minutes after internalization.

RAB-7 was also reported to persist on phagosomes until their degradation (Yu et al. 2008; He et al. 2010), while we observed RAB-7 dissociation around 25 minutes before acidification of the polar body phagosome. These

studies scored phagosome degradation by loss of fluorescent GFP::CED-1 and reported phagosome persistence times of around 55 minutes (He et al. 2010). However, CED-1 is recycled away from the phagosome after internalization and therefore does not reflect phagosome degradation. In case of the 2nd polar body, nearly all CED-1 is lost from the phagosome by the 6-cell stage, at around 7 minutes after internalization (see section 3.3.4), but other fluorescent markers like the mCherry histone marker persist for over 90 minutes. Thus, RAB-7 is recycled away from the phagosome long before its degradation. Also, CED-1 recycling occurs faster than reported for cell corpse phagosomes in later embryonic stages. However, these studies do not report which time point was scored for engulfment and they used different methods to score for fluorescent colocalization on the phagosome.

Thus, recruitment and turnover of the maturation factors RAB-5 and RAB-7 and recycling of the CED-1 receptor appears to occur faster on the polar body phagosome in comparison to cell corpse clearance in later developmental stages. However, to ultimately compare the timing, the same methods should be applied in both cases.

4.5 The role of LC3 during LC3-associated phagocytosis

Colleagues in the lab discovered that depleting an LC3 lipid conjugation factor delayed degradation of the 2nd polar body (Fazeli et al. 2018), emphasizing that lipidated LC3 plays a significant role in degradation of the 2nd polar body. Lab colleagues also showed that polar body degradation is not delayed in autophagy-specific EPG-8 deleted embryos (Fazeli et al. 2018), providing more evidence that the 2nd polar body is degraded via LC3-associated phagocytosis.

As the *C. elegans* LC3 homologues, LGG-1 and LGG-2, filled the polar body phagosome, we questioned whether this could be caused by intrinsic autophagic activity of the polar body. We found that the autophagy-specific EPG-8 was not needed for LC3 localization to the 2nd polar body, while engulfment of the polar body was necessary for LC3 enrichment (see section 3.4.4). So, we concluded that LC3 is recruited to the 2nd polar body phagosome, consistent

with LC3-associated phagocytosis (LAP). These findings open the question how LC3 promotes degradation of the polar body phagosome.

LC3 was proposed to promote autophagosome-lysosome fusion (Ktistakis & Tooze 2016), but our data demonstrated that LC3 lipidation is not needed for the recruitment of LMP-1-positive lysosomes. When we depleted embryos of RAB-7, which is known to promote lysosome-phagosome fusion, we saw a significant delay in LMP-1 recruitment to the polar body phagosome, consistent with previous studies. We also analyzed LMP-1 recruitment to the polar body phagosome in embryos depleted of lipid-conjugated LC3 and found that there was no significant difference in LMP-1 recruitment timing compared to controls (see section 3.4.4.2). Thus, LC3 appears to have another role in LC3-associated phagocytosis than recruiting LMP-1 positive lysosomes.

LC3 has also been proposed to promote the degradation of the inner membrane of autophagosomes (Tsuboyama et al. 2016). Our lab asked whether this could be LC3's role during LAP by specifically labeling the plasma membrane of the polar body using the ZF1 degron technique (Fazeli et al. 2018). Normally they observed a dispersion of the polar body membrane reporter inside the phagosome after 8 minutes. After depleting LC3, polar body membrane dispersion was significantly delayed, suggesting that LC3 promotes membrane breakdown of membranes inside both phagosomes and autophagosomes.

At the same time as the polar body membrane dispersed in the phagosome, the fluorescent histone marker as well as DAPI staining for DNA started to lose their crescent shape and disperse throughout the polar body LAPosome. This observation suggests that phagolysosomal nucleases start degrading the polar body contents after membrane degradation, resulting in free histones and DNA. After membrane breakdown, degradation enzymes should have access to all components of the 2nd polar body to degrade them. However, it takes around 80 more minutes until the fluorescence of an mCherry-tagged histone marker is lost. Depleting LC3 activity prolongs the degradation time even further, thus LC3 is needed for the timely degradation of the 2nd polar body, likely by aiding the degradation of the corpse membrane. It is also intriguing to identify factors

which are involved in later steps of the degradation process, as most research at this point has been focused on early stages of phagsosomes (R. Levin et al. 2016).

Further research is also needed to identify factors that communicate the presence of an inner membrane to recruit LC3. As engulfment receptors recognize membrane components, such as phosphatidylserine on cell corpses and lipopolysaccharides on bacteria (Gong et al. 2011; Chai et al. 2012; Ruggiero et al. 2012), they may initiate a signaling pathway that leads to LC3 recruitment. Engulfment receptors have been shown to lead to the formation of ROS by NADPH oxidase activity (Suh et al. 2006), and intraphagosomal reactive oxygen species (ROS) have been shown to be essential for LC3 recruitment to phagosomes during LAP (J. Huang et al. 2009; Martinez et al. 2015). Thus, engulfment receptors recognizing membrane-wrapped cargo may signal through ROS to recruit LC3, but further research is needed to reveal the signaling pathway.

How LC3 then promotes inner membrane degradation also remains to be investigated. Although we found that LMP-1-positive lysosomes were recruited independent of LC3-lipidation, LC3 could recruit a special subset of lysosomes containing necessary factors needed for the degradation of internal membranes. LC3 could also enable the recruitment of more lysosomes than RAB-7 could alone. Accumulating evidence of the diverse and complex roles of lysosomes in signaling and trafficking suggest that a variety of lysosome subtypes exist and can be recruited by specific factors (Lim & Zoncu 2016). Thus, further research is needed to test whether LC3 promotes corpse membrane degradation by recruiting a subset of lysosomes or through other factors independent of lysosome recruitment.

Assuming LC3 recruits lysosomal subtypes, it remains open how LC3 could attract specialized lysosomes for inner membrane degradation. The pleckstrin homology domain-containing protein family member 1 (PLEKHM1) could be worth investigating in this context as it contains domains that interact with RAB-7 as well as LC3 (McEwan et al. 2015). These could enable PLEKHM1 to

act as an adaptor protein for lysosome fusion of LAPosomes and autophagosomes containing RAB-7 and LC3 on their surface.

Thus, this data highlights the relevance of LC3 in the degradation process of the 2nd polar body. With our data we have revealed its function to degrade the inner membrane of the LAPosome. However, the mechanism of inner membrane degradation remains to be investigated as well as the signaling pathways leading to the recruitment of LC3 to phagocytosed cargo.

4.6 The 2nd polar body as a model for cell death and clearance

C. elegans is a well-established biological model organism (Corsi et al. 2015). All of the discussed cell death and degradation pathways including apoptosis, phagocytosis and LAP are conserved in other species, including mammals (Conradt et al. 2016; Fazeli & Wehman 2017b). Therefore, it is attractive to study them in this model organism, due to *C. elegans'* many advantages for research (see section 1.5).

While many studies focus on the early life of a phagosome (R. Levin et al. 2016), our model system enables researching phagocytosis and degradation from the initial signaling of the dying cell up to its final degradation. The engulfing cells are large in comparison to the corpse, allowing easy tracking of the polar body. We also took advantage of the ZF1 degron technique (Fazeli et al. 2018; Beer et al. 2019), which allows specifically labeling the 2nd polar body in a dark background, making it easy to track until its final degradation. Polar body clearance takes around 90 minutes, making quantitative time-lapse studies during the whole process feasible. All these advantages allowed us to put this whole process from the initial signaling until degradation of the 2nd polar body into a timely perspective (see Fig. 22) to improve our understanding of the process and to facilitate comparisons after protein knockdowns.

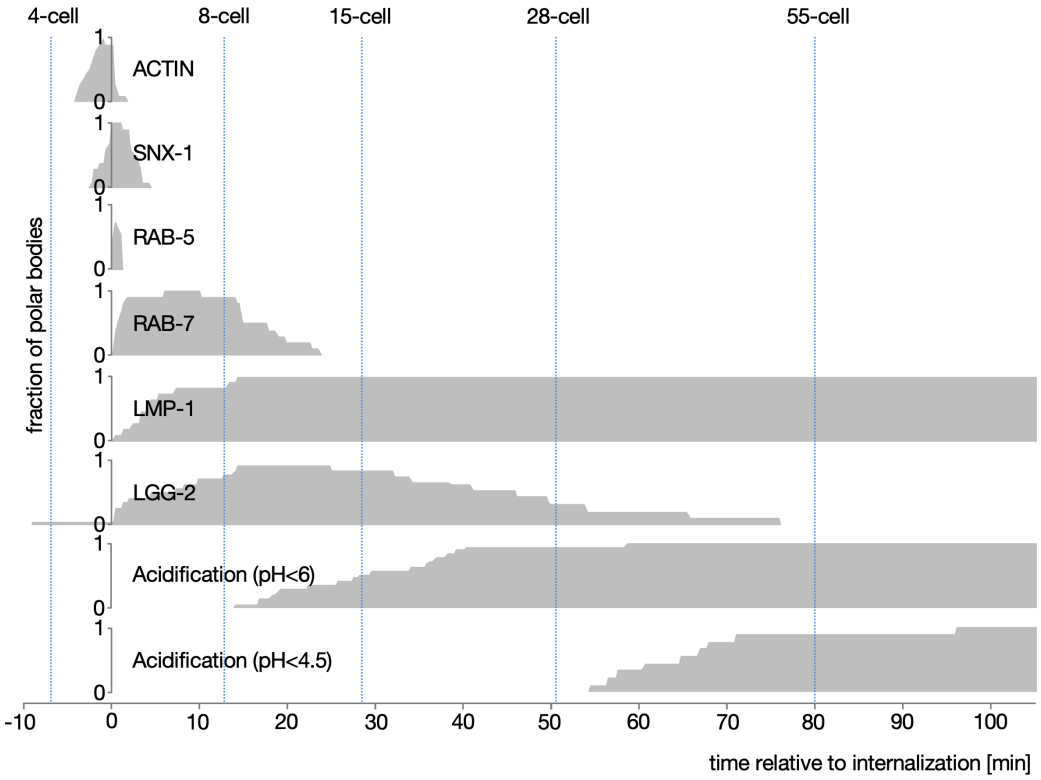


Fig. 22 – Using fluorescent time lapse imaging, we were able to put polar body internalization, phagosome maturation and degradation in a timely perspective.
Actin accumulates prior to polar body internalization. SNX-1 can be found on the phagosome, which likely promotes further phagosome maturation, including the recruitment of RAB-5, which is quickly exchanged for RAB-7. RAB-7 promotes the fusion with LMP-1-positive lysosomes. The polar body phagosome is degraded by LC3-associated phagocytosis, therefore also recruiting the LC3 homologue LGG-2 to the phagosome. Lysosomes lead to further acidification und ultimately the degradation of the polar body. With these scorings we put internalization and phagosome maturation in a timely perspective and were able to compare recruitment of various factors and acidification after genetic modifications, making the 2nd polar body an attractive model to study cell death and clearance by LC3-associated phagocytosis.
Blue lines indicate cell stages for orientation assuming internalization of the 2nd polar body happened during the 4-cell stage.

4.7 LC3-associated phagocytosis in humans and disease

Despite the recent discovery of LC3-associated phagocytosis (LAP), growing evidence has demonstrated its involvement in a variety of processes (Heckmann & D. R. Green 2019). As LAP is an evolutionary conserved process, investigating it in a model organism such as *C. elegans* will improve our understanding and further reveal its importance in humans.

A part of LAP's role is to clear the extracellular space from infectious agents, controlling an infection. Mice lacking LAP have reduced clearing of fungal infections and infections with *Listeria monocytogenes, Legionella dumoffii, Toxoplasma gondi* and *Helicobacter pylori* (Martinez et al. 2015; Deen et al. 2015; Florey et al. 2015; Oikonomou et al. 2018). It has also been shown to regulate the inflammatory response by leading to a type-I interferon response, important for a host defense against pathogens (Hayashi et al. 2018).

But besides its pro-inflammatory role in defending against pathogens, LAP also acts as an anti-inflammatory factor (Heckmann & D. R. Green 2019). Mice lacking LAP show accumulation of apoptotic bodies in their tissue and phagocytic cells (Muñoz et al. 2010), and defects of LC3 in mice have been demonstrated to result in a systemic lupus erythematosus like disease (Martinez et al. 2016). This demonstrates that LAP has a dual function in promoting pro- and anti-inflammatory responses. Its anti-inflammatory role also has its downsides though, as it is possible to hinder an effective immune response to cancerous cells by disrupting LAP (Heckmann & D. R. Green 2019). On the other hand, this poses an attractive target for novel cancer treatment strategies, as hindering LAP can promote an immune response against the tumor (Cunha et al. 2018).

LAP's role in cell homeostasis, inflammation, autoimmunity and pathogen clearance underlines its significance in human health. It poses an attractive target for novel therapies and depending on the circumstances it can likely be manipulated to either be enhanced or inhibited. Thus, research on LAP is promising to investigate in future work.

With our work, we have established an *in vivo* model to study cell death, phagosome maturation and clearance by LC3-associated phagocytosis (LAP) using the *C. elegans* 2nd polar body.

5 CONCLUSION

It was our goal to characterize polar body death and clearance as they are basic biological processes, which are evolutionarily conserved in animals, including mammals. Polar bodies are a product of female meiosis. In *C. elegans,* the extrusion of the 1st polar body happens during eggshell formation, which leaves it trapped in eggshell layers with no contact to the embryo. The 2nd polar body ends up in contact with embryonic cells and gets internalized by the embryo. Although it was known that they both die, their dying mechanism as well as their fate being cleared by embryonic cells has not been well understood. The 2nd polar body appeared to be an attractive model to study corpse clearance as it is engulfed by rather big cells, therefore easy to track by fluorescence microscopy even until ultimate degradation by using the ZF1-degron technique.

We asked whether polar bodies could become necrotic and therefore harm the embryo with the release of their intracellular content. I demonstrated that both polar bodies quickly lose membrane integrity by staining them with propidium iodide (PI), indicating they undergo a form of accelerated necrosis, as it occurred faster than secondary necrosis. To show necrosis of the 2nd polar body, I had to disrupt the eggshell's permeability barrier by knocking down PTR-2, since PI was not able to cross it. Since it has been shown that the death of both polar bodies also has apoptotic features to it and since the cells are likely still in a meiotic program, I suggested to call this yet uncharacterized type of cell death "meioptosis".

As the 1st polar body is trapped in eggshell layers, we wondered whether it would stay there and asked where it ends up after the worm hatches. By time-lapse imaging I confirmed it remains within the eggshell throughout embryogenesis and can be found in remnants of the eggshell after the worm hatches, which could be beneficial for the embryo as it avoids contact with this necrotic cell.

However, the 2nd polar body is internalized by its neighboring cell before the embryo reaches the 6-cell stage. Before and shortly after internalization CED-1 accumulates on the 2nd polar body, dependent on SNX-6 activity. The polar body is internalized in a CED-10 dependent manner, either by ABa- or the

ABp-cell as it often is dragged in between the two cells during the 3-cell division. This characteristic movement is promoted by RAB-5 and VPS-34 activity, but adhesion factors are left to be identified. We have observed internalization by other cells and colleagues showed polar body inheritance was random, thus making polar body internalization unlikely to influence the cell's fate.

After internalization, the polar body phagosome quickly recruits the early endosome marker RAB-5, which is then exchanged for the late endosome marker RAB-7. RAB-7 also dissolves from the phagosome but is needed for recruitment of LMP-1 positive lysosomes and the timely degradation of the 2nd polar body. LMP-1 persists on the phagosome, which further acidifies up until the ultimate degradation of the 2nd polar body, which takes around 90 minutes.

As it was reported that dying cells are cleared by LC3-associated phagocytosis (LAP), we also asked whether the 2nd polar body would be cleared by this mechanism. I confirmed that both *C. elegans* LC3 homologues, LGG-1 and LGG-2, indeed colocalize with the phagosome shortly after internalization and persist for around 40 minutes. Since it appeared to fill the polar body rather than localize on its membrane, we asked whether this activity was seen due to an increased intrinsic autophagic activity. But we saw no difference after deleting the autophagy-specific protein EPG-8, but blocking internalization did indeed reduce LC3 colocalization significantly, suggesting LC3 activity is caused by phagocytosis, not autophagy. Thus, the 2nd polar body is degraded via LC3-associated phagocytosis.

LC3 depletion delayed polar body degradation, so I tested whether that would be caused by a delayed recruitment of lysosomes. However, there was no significant difference after LC3 depletion. Instead, colleagues in our lab have demonstrated that it promotes degradation of the corpse membrane instead, revealing LC3's role in LAP. However, it remains to be investigated how it is communicated when membrane-wrapped cargo is internalized and how LC3 aids in membrane degradation.

With this work, we have established the 2nd polar body as a model to study cell death and clearance by LC3-associated phagocytosis (LAP, see Fig. 23).

LAP has been shown to be a key process in cell homeostasis, inflammation, autoimmunity and pathogen clearance and thereby promoting health but also disease. Thus, this field will be promising to explore in future publications and the 2nd polar body poses an attractive research object in a well-established model organism *Caenorhabditis elegans*.

6 ZUSAMMENFASSUNG

Unser Ziel war es den Zelltod der Polkörper und ihren Abbauprozess näher zu untersuchen, nachdem beide Prozesse grundlegende biologische Zellfunktionen darstellen, die evolutionär zwischen vielen Spezies, einschließlich Säugetieren, konserviert sind. Polkörper sind ein Produkt der weiblichen Meiose. In *C. elegans* erfolgt die Bildung der 1. Polkörpers zeitgleich mit der Bildung der Eischale, was dazu führt, dass er in Schichten dieser gefangen ist. Der zweite Polkörper verbleibt hingegen unter der Eischale in Kontakt zu embryonalen Zellen und wird von diesen internalisiert. Der 2. Polkörper erschien uns dabei als attraktives Studienobjekt, weil es von verhältnismäßig großen Zellen internalisiert wird, was eine fluoreszenzmikroskopische Nachverfolgung ermöglicht, mit der ZF1-Degron Technik sogar bis zum endgültigen Abbau des Polkörpers.

Wir fragten uns ob Polkörper nekrotisch werden, wodurch sie den Embryo durch die Freisetzung des Zellinhalts schädigen könnten. Ich färbte die Polkörper mit Propidiumiodid (PI) und zeigte dadurch, dass beide ihre Membranintegrität verlieren. Dieses Zeichen von Nekrose trat recht schnell auf, ist also eher als Zeichen einer beschleunigten als einer sekundären Nekrose zu werten. Um dies zu zeigen musste ich die Permeabilitätsbarriere der Eischale durch Ausschalten von PTR-2 zerstören, da PI durch diese nicht diffundieren konnte. Da gezeigt wurde, dass der Tod der Polkörper auch Charakteristika von Apoptose ausweist und weil sich die Polkörper wahrscheinlich noch in einem meiotischen Zustand befinden, schlage ich vor diesen bisher uncharakterisierten Zelltod als „Meioptose“ zu bezeichnen.

Der erste Polkörper ist in Schichten der Eischale gefangen, daher fragten wir uns ob dies während der gesamten Embryonalentwicklung der Fall sei und

was nach dem Schlüpfen des Embryos mit ihm geschieht. In einer Zeitrafferaufnahme zeigte sich, dass er während der gesamten Entwicklung unbewegt in der Eischale verbleibt und nach dem Schlüpfen der Larve in Überresten dieser gefunden werden kann, wodurch der Embryo möglicherweise einen Kontakt mit der nekrotischen Zelle vermeidet.

Der zweite Polkörper wird allerdings von benachbarten embryonalen Zellen internalisiert, bevor der Embryo das 6-Zell Stadium erreicht hat. Vor und kurz nach seiner Internalisierung zeigt sich eine Akkumulation von CED-1 auf dem Polkörper. CED-1 Akkumulation ist abgängig von SNX-6 Aktivität. Die Internalisierung selbst ist abhängig von CED-10 Aktivität und erfolgt entweder von der ABa- oder der ABp-Zelle, zwischen die der Polkörper häufig während der Drei-Zell-Teilung hineingezogen wird. Diese charakteristische Bewegung wird von RAB-5 und VPS-34 gefördert. Faktoren, die direkt an den Polkörper binden, müssen jedoch noch identifiziert werden. Die Internalisierung kann auch von jeder anderen Zelle als nur von ABa und ABp erfolgen und Laborkollegen zeigten, dass die Vererbung des Polkörpers nach Internalisierung zufällig erfolgte. Daher ist es eher unwahrscheinlich, dass der Polkörper einen Einfluss auf die Zelldifferenzierung hat.

Kurz nach der Internalisierung wird der Marker für frühe Endosomen RAB-5 rekrutiert, der kurze Zeit später für den Marker für späte Endosomen RAB-7 ausgetauscht wird. RAB-7 rekrutiert LMP-1-positive Lysosomen und wird für den zeitgerechten Abbau des Polkörper benötigt. RAB-7 löst sich wieder vom Phagosom, während das Phagosom während des gesamten Abbaus LMP-1 positiv bleibt. Die Lysosomen sorgen für eine ansteigende Ansäuerung, sodass der Abbau des Polkörpers circa 90 Minuten dauert.

Es war bereits bekannt, dass sterbende Zellen durch LC3-assoziierte Phagozytose (LAP) abgebaut werden. Wir stellten daher die Hypothese auf, dass dies beim 2. Polkörper ebenfalls der Fall sei. Ich bestätigte, dass beide LC3 homologen Proteine, LGG-1 und LGG-2, kurz nach Internalisierung für etwa 40 Minuten auf dem Phagosom gefunden werden können. Es schien jedoch so, als ob sie eher das Phagosom ausfüllten, als zur Membran rekrutiert zu werden. Wir

fragten uns daher, ob die LC3 Aktivität durch intrinsische Autophagie des Polkörpers verursacht werden könnte und führten eine Deletion des Autophagie-spezifischen Gens *epg-8* durch. Dies führte zu keiner signifikanten Veränderung des Phänotyps, aber eine Blockierung der Internalisierung über *ced-1* Knockdown zeigte signifikant niedrigere LC3 Kolokalisationen mit dem Polkörper. Daraus ist zu schließen, dass die Präsenz von LC3 auf LAP und nicht auf Autophagie zurückzuführen ist. Daher wird der 2. Polkörper über LC3-assoziierte Phagozytose abgebaut.

LC3 Knockdown hat zu einem verlangsamten Abbau des Polkörpers geführt, daher testete ich, ob dies mit einer verspäteten Rekrutierung von Lysosomen zusammenhängen könnte. Allerdings zeigte sich nach einer entsprechenden RNA-Interfenz keine Veränderung in der Rekrutierung von Lysosomen. Kollegen aus dem Labor zeigten mithilfe der ZF1-Degron Technik, dass ein LC3-Knockdown einen verspäteten Abbau der Membran des Polkörpers innerhalb des Phagosoms bewirkt. Dadurch zeigten wir zum ersten Mal, dass die Rolle von LC3 in LAP im Abbau der inneren Membran liegt. Es bleibt allerdings offen, wie die Präsenz einer Membran um das internalisierte Material kommuniziert wird und wie LC3 deren Abbau fördert.

Mit dieser Arbeit etablierten wir den 2. Polkörper als ein biologisches Modell um Zelltod und Abbau über LC3-assoziierte Phagozytose (LAP, s. Abb. 23) zu erforschen. Die Rolle der LAP in Prozessen der Zell-Homöostase, Entzündung, Autoimmunität und dem Abbau von pathogenen Erregern und damit als Faktor, der sowohl Gesundheit als auch Krankheit fördern kann, ist mittlerweile bekannt. Daher wird es in diesem Feld in Zukunft versprechende Forschung geben. Mit dem 2. Polkörper haben wir ein attraktives Forschungsobjekt in dem bereits hervorragend charakterisierten Modellorganismus *Caenorhabditis elegans* geschaffen.

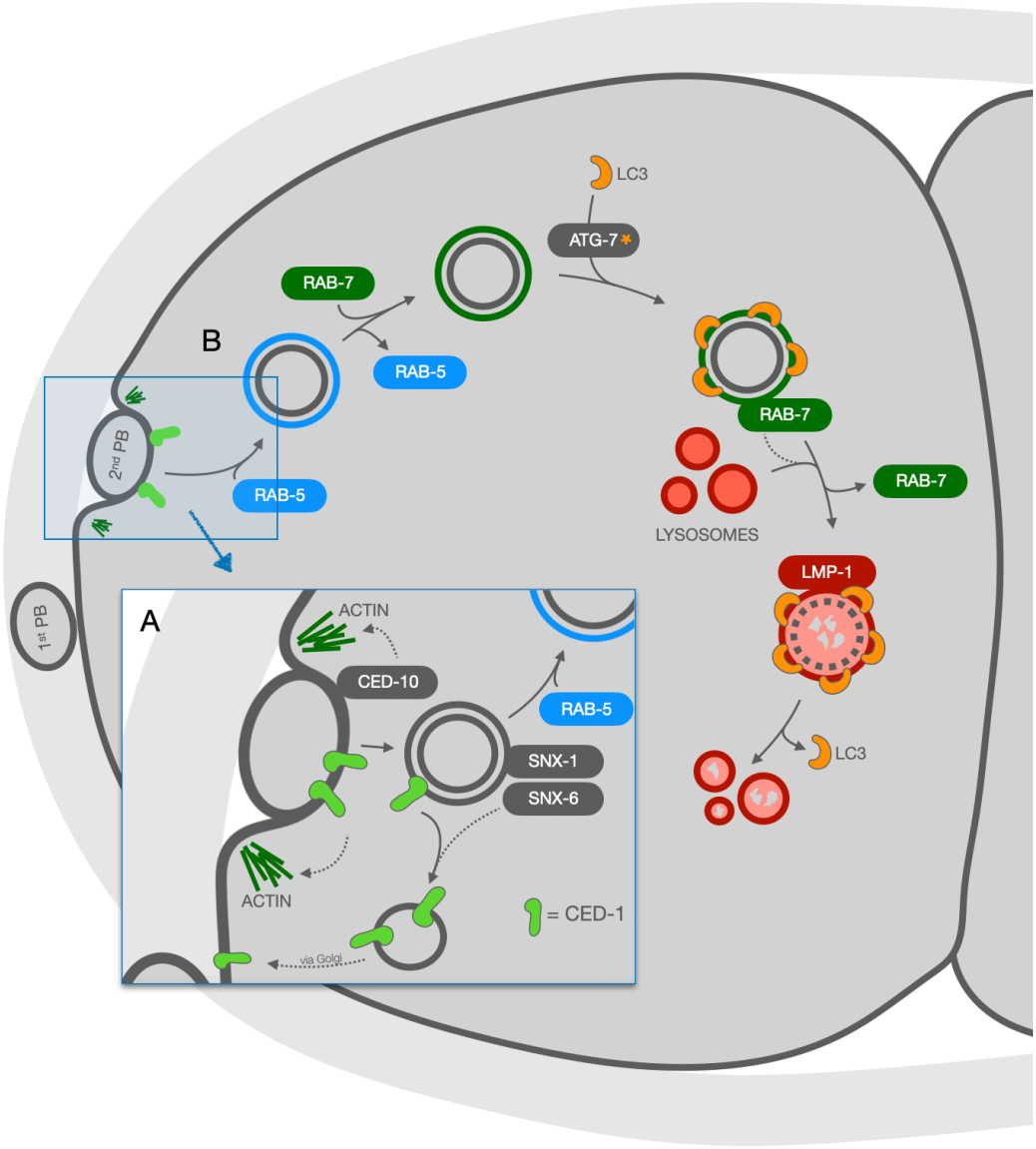


Fig. 23 – The internalization and degradation of the 2nd polar body via LC3-associated phagocytosis. Concluding figure showing the anterior fraction of a 2-cell stage embryo. The 1st polar body (1st PB) can be found trapped in the eggshell (light grey, around the embryo), while the 2nd polar body (2nd PB) has contact with the embryo. In that case it is internalized by the anterior blastomere (AB) cell and degraded via LC3-associated phagocytosis.
(A) The 2nd polar body quickly dies after its extrusion. It presents "eat-me" signals for internalization (not shown). CED-1 receptor as well as the CED-10/Rac pathway are needed for the internalization and rearrangement of actin filaments, engulfing the 2nd polar body. CED-1 is recycled away from the phagosome by SNX-6 activity via the Golgi apparatus back to the plasma membrane. Its heterodimer SNX-1 can be found on the phagosome, which is known to promote further phagosome maturation.
(B) The polar body phagosome quickly recruits RAB-5, which is exchanged for RAB-7 within a minute. ATG-7 lipidates LC3, which can then be found on the phagosome. RAB-7 promotes the fusion of lysosomes with the polar body phagosome, leading to further acidification. Both RAB-7 and LC3 dissociate from the phagosome. Degradation of the polar body involves vesiculation and takes around 90 minutes.
The embryo and the polar body are drawn to scale before its internalization. Dotted arrows show promotion of a process.

Abb. 23 – Die Internalisierung und der Abbau des 2. Polkörpers mittels LC3-assoziierter Phagozytose.
Diese zusammenfassende Abbildung zeigt den anterioren Abschnitt des Embryos im Zweizellstadium. Der erste Polkörper (1st PB) findet sich gefangen in der Eischale (hellgraue Ummantelung des Embryos). Der zweite Polkörper (2nd PB) hat hingegen Kontakt mit dem Embryo und wird in diesem Fall von der anterioren Blastomere (AB) internalisiert und mittels LC3-assocziierter Phagozytose abgebaut.
(A) Der 2. Polkörper stirbt schnell nach seiner Bildung. Er präsentiert „friss-mich"-Signale, um eine Internalisierung zu fördern (nicht gezeigt). Der Rezeptor CED-1, wie auch der CED-10/Rac Signalweg sind erforderlich für die Internalisierung des 2. Polkörpers durch eine Umstrukturierung von Aktin. CED-1 wird mithilfe von SNX-6 über den Golgi-Apparat zurück zur Plasmamembran recycelt. Sein Heterodimer SNX-1 findet sich ebenfalls auf dem Phagosom, der die weitere Reifung des Phagosoms fördert.
(B) Das Polkörper-Phagosom rekrutiert kurz nach seiner Internalisierung RAB-5, welches für RAB-7 innerhalb von einer Minute ausgetauscht wird. ATG-7 lipidiert LC3, was eine Bindung an das Phagosom ermöglicht. RAB-7 fördert die Fusion von Lysosomen mit dem Phagosom, was zu einer weiteren Ansäuerung führt. Sowohl RAB-7 als auch LC3 lösen sich wieder vom Phagosom, welches nach mehrfacher Vesikulation nach etwa 90 Minuten abgebaut wird.
Der Embryo und die Polkörper sind zueinander vor der Internalisierung maßstabsgetreu gezeichnet. Gestrichelte Pfeile stellen fördernden Einfluss auf einen Prozess dar.

7 REFERENCES

Adams, J.M. & Cory, S., 2001. Life-or-death decisions by the Bcl-2 protein family. *Trends in Biochemical Sciences*, 26(1), pp.61–66.

Albert, M.L. et al., 1998. Immature Dendritic Cells Phagocytose Apoptotic Cells via α vβ 5and CD36, and Cross-present Antigens to Cytotoxic T Lymphocytes. *The Journal of Experimental Medicine*, 188(7), pp.1359–1368.

Amer, A.O. & Swanson, M.S., 2005. Autophagy is an immediate macrophage response to Legionella pneumophila. *Cellular Microbiology*, 7(6), pp.765–778.

Bachmann, M. et al., 2019. Cell Adhesion by Integrins. *Physiological Reviews*, 99(4), pp.1655–1699.

Balla, T. & Várnai, P., 2009. Visualization of Cellular Phosphoinositide Pools with GFP-Fused Protein-Domains J. S. Bonifacino et al., eds. *Current Protocols in Cell Biology*, 42(1), pp.24.4.1–24.4.27.

Beer, K.B. et al., 2019. Degron-tagged reporters probe membrane topology and enable the specific labelling of membrane-wrapped structures. *Nature Communications*, 10(1), pp.1–16.

Beer, K.B. et al., 2018. Extracellular vesicle budding is inhibited by redundant regulators of TAT-5 flippase localization and phospholipid asymmetry. *Proceedings of the National Academy of Sciences of the United States of America*, 115(6), pp.E1127–E1136.

Bellone, M. et al., 1997. Processing of engulfed apoptotic bodies yields T cell epitopes. *The Journal of Immunology*, 159(11), pp.5391–5399.

Belz, G.T. et al., 2002. The CD8α +Dendritic Cell Is Responsible for Inducing Peripheral Self-Tolerance to Tissue-associated Antigens. *The Journal of Experimental Medicine*, 196(8), pp.1099–1104.

Benchimol, S., 2001. p53-dependent pathways of apoptosis. *Cell death and differentiation*, 8(11), pp.1049–1051.

Benenati, G. et al., 2009. Two cytochrome P450s in Caenorhabditis elegans are essential for the organization of eggshell, correct execution of meiosis and the polarization of embryo. *Mechanisms of development*, 126(5-6), pp.382–393.

Bieber, F.R. et al., 1981. Genetic studies of an acardiac monster: evidence of polar body twinning in man. *Science (New York, N.Y.)*, 213(4509), pp.775–777.

Bondanza, A. et al., 2004. Inhibition of Phosphatidylserine Recognition Heightens the Immunogenicity of Irradiated Lymphoma Cells In Vivo. *The Journal of Experimental Medicine*, 200(9), pp.1157–1165.

Botto, M. & Walport, M.J., 2002. C1q, autoimmunity and apoptosis. *Immunobiology*, 205(4-5), pp.395–406.

Bratton, D.L. et al., 1997. Appearance of phosphatidylserine on apoptotic cells requires calcium-mediated nonspecific flip-flop and is enhanced by loss of the aminophospholipid translocase. *The Journal of biological chemistry*, 272(42), pp.26159–26165.

Brenner, S., 2003. NOBEL LECTURE: Nature's Gift to Science. *Biosci Rep*, 23(5-6), p.225.

Brenner, S., 1974. The genetics of Caenorhabditis elegans. *Genetics*, 77(1), pp.71–94.

Brown, G.C. & Neher, J.J., 2012. Eaten alive! Cell death by primary phagocytosis: “phagoptosis.” *Trends in Biochemical Sciences*, 37(8), pp.325–332.

Brugnera, E. et al., 2002. Unconventional Rac-GEF activity is mediated through the Dock180–ELMO complex. *Nature cell biology*, 4(8), pp.574–582.

Brunner, T. et al., 2003. Fas (CD95/Apo-1) ligand regulation in T cell homeostasis, cell-mediated cytotoxicity and immune pathology. *Seminars in immunology*, 15(3), pp.167–176.

Cabello, J. et al., 2010. The Wnt Pathway Controls Cell Death Engulfment, Spindle Orientation, and Migration through CED-10/Rac B. Conradt, ed. *PLoS Biology*, 8(2), pp.e1000297–12.

Campbell, K.J. & Tait, S.W.G., 2018. Targeting BCL-2 regulated apoptosis in cancer. *Open Biology*, 8(5), pp.180002–11.

Carvalho, A. et al., 2011. Acute drug treatment in the early C. elegans embryo. B. Lehner, ed. *PloS one*, 6(9), p.e24656.

Casares, N. et al., 2005. Caspase-dependent immunogenicity of doxorubicin-induced tumor cell death. *The Journal of Experimental Medicine*, 202(12), pp.1691–1701.

Chai, Y. et al., 2012. Apoptotic regulators promote cytokinetic midbody degradation in C. elegans. *The Journal of cell biology*, 199(7), pp.1047–1055.

Chalfie, M., 2009. *GFP: Lighting up life*, National Academy of Sciences.

Chalfie, M. et al., 1994. Green fluorescent protein as a marker for gene expression. *Science (New York, N.Y.)*, 263(5148), pp.802–805.

Chen, D. et al., 2010. Retromer is required for apoptotic cell clearance by phagocytic receptor recycling. *Science (New York, N.Y.)*, 327(5970), pp.1261–1264.

Chimini, G. & Chavrier, P., 2000. Function of Rho family proteins in actin dynamics during phagocytosis and engulfment. *Nature cell biology*, 2(10), pp.E191–6.

Conradt, B., Wu, Y.-C. & Xue, D., 2016. Programmed Cell Death During Caenorhabditis elegans Development. *Genetics*, 203(4), pp.1533–1562.

Corsi, A.K., Wightman, B. & Chalfie, M., 2015. A Transparent Window into Biology: A Primer on Caenorhabditis elegans. *Genetics*, 200(2), pp.387–407.

Coudreuse, D.Y.M. et al., 2006. Wnt Gradient Formation Requires Retromer Function in Wnt-Producing Cells. *Science (New York, N.Y.)*, 312(5775), pp.921–924.

Coussens, L.M. & Werb, Z., 2002. Inflammation and cancer. *Nature*, 420(6917), pp.860–867.

Crittenden, S.L. et al., 2003. Regulation of the mitosis/meiosis decision in the Caenorhabditis elegansgermline. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 358(1436), pp.1359–1362.

Cunha, L.D. et al., 2018. LC3-Associated Phagocytosis in Myeloid Cells Promotes Tumor Immune Tolerance. *Cell*, 175(2), pp.429–441.e16.

Dalton, C.M. & Carroll, J., 2013. Biased inheritance of mitochondria during asymmetric cell division in the mouse oocyte. *Journal of cell science*, 126(13), pp.2955–2964.

Dangi, A., Yu, S. & Luo, X., 2018. Apoptotic cell-based therapies for promoting transplantation tolerance. *Current Opinion in Organ Transplantation*, pp.1–7.

Davis, D.E. et al., 2009. The cation diffusion facilitator gene cdf-2 mediates zinc metabolism in Caenorhabditis elegans. *Genetics*, 182(4), pp.1015–1033.

Deen, N.S. et al., 2015. Analysis of the Relative Contribution of Phagocytosis, LC3-Associated Phagocytosis, and Canonical Autophagy During Helicobacter pyloriInfection of Macrophages. *Helicobacter*, 20(6), pp.449–459.

Desmyttere, S. et al., 2009. Growth and health outcome of 102 2-year-old children conceived after preimplantation genetic diagnosis or screening. *Early Human Development*, 85(12), pp.755–759.

Devitt, A. et al., 2004. Persistence of apoptotic cells without autoimmune disease or inflammation in CD14 –/–mice. *The Journal of cell biology*, 167(6), pp.1161–1170.

Doherty, G.J. & McMahon, H.T., 2008. Mediation, Modulation, and Consequences of Membrane-Cytoskeleton Interactions. *Annual Review of Biophysics*, 37(1), pp.65–95.

Dong, T. et al., 2015. Using Small Molecules to Dissect Non-apoptotic Programmed Cell Death: Necroptosis, Ferroptosis, and Pyroptosis. *ChemBioChem*, 16(18), pp.2557–2561.

Dorn, J.F. et al., 2010. Actomyosin Tube Formation in Polar Body Cytokinesis Requires Anillin in C. elegans. *Current Biology*, 20(22), pp.2046–2051.

Dunn, G.P., Koebel, C.M. & Schreiber, R.D., 2006. Interferons, immunity and cancer immunoediting. *Nature reviews. Immunology*, 6(11), pp.836–848.

Eisenhut, R.J., Knox, D. & Hermann, G.J., 2005. Characterization of a conserved apoptotic marker expressed in Caenorhabditis elegans phagocytic cells. *Biochemical and Biophysical Research Communications*, 335(4), pp.1231–1238.

Elliott, M.R. & Ravichandran, K.S., 2016. The Dynamics of Apoptotic Cell Clearance. *Developmental cell*, 38(2), pp.147–160.

Ellis, H.M. & Horvitz, H.R., 1986. Genetic control of programmed cell death in the nematode C. elegans. *Cell*, 44(6), pp.817–829.

Ellis, R.E. & Horvitz, H.R., 1991. Two C. elegans genes control the programmed deaths of specific cells in the pharynx. *Development (Cambridge, England)*, 112(2), pp.591–603.

Elmore, S., 2007. Apoptosis: a review of programmed cell death. *Toxicologic pathology*, 35(4), pp.495–516.

Eskelinen, E.-L., Tanaka, Y. & Saftig, P., 2003. At the acidic edge: emerging functions for lysosomal membrane proteins. *Trends in cell biology*, 13(3), pp.137–145.

Fabian, D. et al., 2012. Do embryonic polar bodies commit suicide? *Zygote*, 22(01), pp.10–17.

Fabritius, A.S., Ellefson, M.L. & McNally, F.J., 2011. Nuclear and spindle positioning during oocyte meiosis. *Current opinion in cell biology*, 23(1), pp.78–84.

Fadok, V.A. et al., 2001. Loss of Phospholipid Asymmetry and Surface Exposure of Phosphatidylserine Is Required for Phagocytosis of Apoptotic Cells by Macrophages and Fibroblasts. *The Journal of biological chemistry*, 276(2), pp.1071–1077.

Fairn, G.D. & Grinstein, S., 2012. How nascent phagosomes mature to become phagolysosomes. *Trends in Immunology*, 33(8), pp.397–405.

Fazeli, G. & Wehman, A.M., 2017a. Rab GTPases mature the LC3-associated midbody phagosome. *Communicative & Integrative Biology*, 10(2), p.e1297349.

Fazeli, G. & Wehman, A.M., 2017b. Safely removing cell debris with LC3-associated phagocytosis. *Biology of the cell*, 109(10), pp.355–363.

Fazeli, G. et al., 2018. C. elegans Blastomeres Clear the Corpse of the Second Polar Body by LC3-Associated Phagocytosis. *CellReports*, 23(7), pp.2070–2082.

Fazeli, G. et al., 2016. C. elegans midbodies are released, phagocytosed and undergo LC3-dependent degradation independent of macroautophagy. *Journal of cell science*, 129(20), pp.3721–3731.

Feinberg, E.H. et al., 2008. GFP Reconstitution Across Synaptic Partners (GRASP) Defines Cell Contacts and Synapses in Living Nervous Systems. *Neuron*, 57(3), pp.353–363.

Fiers, W. et al., 1999. More than one way to die: apoptosis, necrosis and reactive oxygen damage. *Oncogene*, 18(54), pp.7719–7730.

Fire, A.Z., 2007. Gene Silencing by Double-Stranded RNA (Nobel Lecture). *Angewandte Chemie International Edition*, 46(37), pp.6966–6984.

Flannagan, R.S., Jaumouillé, V. & Grinstein, S., 2012. The cell biology of phagocytosis. *Annual review of pathology*, 7(1), pp.61–98.

Florey, O. et al., 2011. Autophagy machinery mediates macroendocytic processing and entotic cell death by targeting single membranes. *Nature Publishing Group*, 13(11), pp.1335–1343.

Florey, O. et al., 2015. V-ATPase and osmotic imbalances activate endolysosomal LC3 lipidation. *Autophagy*, 11(1), pp.88–99.

Fraser, A.G. et al., 2000. Functional genomic analysis of C. elegans chromosome I by systematic RNA interference. *Nature*, 408(6810), pp.325–330.

Fujiwara, Y., Wada, K. & Kabuta, T., 2016. Lysosomal degradation of intracellular nucleic acids—multiple autophagic pathways. *Journal of Biochemistry*, 17, pp.mvw085–10.

Galluzzi, L. et al., 2008. To die or not to die: that is the autophagic question. *Current molecular medicine*, 8(2), pp.78–91.

Gitlin, S.A., Gibbons, W.E. & Gosden, R.G., 2003. Oocyte biology and genetics revelations from polar bodies. *Reproductive BioMedicine Online*, 6(4), pp.403–409.

Gong, L. et al., 2011. The Burkholderia pseudomallei Type III Secretion System and BopA Are Required for Evasion of LC3-Associated Phagocytosis. *PloS one*, 6(3), p.e17852.

Gonzales, D.S., Bavister, B.D. & Mese, S.A., 2001. In utero and in vitro proteinase activity during the Mesocricetus auratus embryo zona escape time window. *Biology of reproduction*, 64(1), pp.222–230.

Gozuacik, D. & Kimchi, A., 2004. Autophagy as a cell death and tumor suppressor mechanism. *Oncogene*, 23(16), pp.2891–2906.

Green, R.A. et al., 2008. Expression and imaging of fluorescent proteins in the C. elegans gonad and early embryo. *Methods in cell biology*, 85, pp.179–218.

Gregory, C., 2009. Cell biology: Sent by the scent of death. *Nature*, 461(7261), pp.181–182.

Gregory, C.D. & Pound, J.D., 2010. Cell death in the neighbourhood: direct microenvironmental effects of apoptosis in normal and neoplastic tissues M. Ladanyi & P. C. Hogendoorn, eds., 223(2), pp.178–195.

Gumienny, T.L. et al., 2001. CED-12/ELMO, a novel member of the CrkII/Dock180/Rac pathway, is required for phagocytosis and cell migration. *Cell*, 107(1), pp.27–41.

Gumienny, T.L. et al., 1999. Genetic control of programmed cell death in the Caenorhabditis elegans hermaphrodite germline. *Development (Cambridge, England)*, 126(5), pp.1011–1022.

Guo, P. et al., 2010. Sequential action of Caenorhabditis elegans Rab GTPases regulates phagolysosome formation during apoptotic cell degradation. *Proceedings of the National Academy of Sciences of the United States of America*, 107(42), pp.18016–18021.

Hammoud, I. et al., 2010. Are zona pellucida laser drilling and polar body biopsy safe for in vitro matured oocytes? *Journal of Assisted Reproduction and Genetics*, 27(7), pp.423–427.

Hamon, Y. et al., 2006. Cooperation between Engulfment Receptors: The Case of ABCA1 and MEGF10 R. Insall, ed. *PloS one*, 1(1), pp.e120–9.

Han, C.Z. & Ravichandran, K.S., 2011. Metabolic connections during apoptotic cell engulfment. *Cell*, 147(7), pp.1442–1445.

Hanayama, R. et al., 2002. Identification of a factor that links apoptotic cells to phagocytes. *Nature*, 417(6885), pp.182–187.

Harrison, R.E. et al., 2003. Phagosomes Fuse with Late Endosomes and/or Lysosomes by Extension of Membrane Protrusions along Microtubules: Role of Rab7 and RILP. *Molecular and Cellular Biology*, 23(18), pp.6494–6506.

Haslett, C., 1999. Granulocyte apoptosis and its role in the resolution and control of lung inflammation. *American Journal of Respiratory and Critical Care Medicine*, 160(5 Pt 2), pp.S5–11.

Hayashi, K., Taura, M. & Iwasaki, A., 2018. The interaction between IKKα and LC3 promotes type I interferon production through the TLR9-containing LAPosome. *Science signaling*, 11(528).

He, B. et al., 2010. Live-cell imaging in Caenorhabditis elegans reveals the distinct roles of dynamin self-assembly and guanosine triphosphate hydrolysis in the removal of apoptotic cells. *Molecular biology of the cell*, 21(4), pp.610–629.

Heckmann, B.L. & Green, D.R., 2019. LC3-associated phagocytosis at a glance. *Journal of cell science*, 132(5).

Hengartner, M.O. & Horvitz, H.R., 1994. C. elegans cell survival gene ced-9 encodes a functional homolog of the mammalian proto-oncogene bcl-2. *Cell*, 76(4), pp.665–676.

Hengartner, M.O., Ellis, R.E. & Horvitz, H.R., 1992. Caenorhabditis elegans gene ced-9 protects cells from programmed cell death. *Nature*, 356(6369), pp.494–499.

Herrmann, M. et al., 1998. Impaired phagocytosis of apoptotic cell material by monocyte-derived macrophages from patients with systemic lupus erythematosus. *Arthritis and rheumatism*, 41(7), pp.1241–1250.

Hochreiter-Hufford, A. & Ravichandran, K.S., 2013. Clearing the dead: apoptotic cell sensing, recognition, engulfment, and digestion. *Cold Spring Harbor Perspectives in Biology*, 5(1), pp.a008748–a008748.

Hodge, S. et al., 2003. Alveolar macrophages from subjects with chronic obstructive pulmonary disease are deficient in their ability to phagocytose apoptotic airway epithelial cells. *Immunology and cell biology*, 81(4), pp.289–296.

Hodgkin, J., 2001. What does a worm want with 20,000 genes? *Genome biology*, 2(11), pp.COMMENT2008–4.

Hoeppner, D.J., Hengartner, M.O. & Schnabel, R., 2001. Engulfment genes cooperate with ced-3 to promote cell death in Caenorhabditis elegans. *Nature*, 412(6843), pp.202–206.

Horvitz, H.R., 1999. Genetic control of programmed cell death in the nematode Caenorhabditis elegans. *Cancer research*, 59(7 Suppl), pp.1701s–1706s.

Horvitz, H.R., 2003. Worms, Life, and Death (Nobel Lecture). *ChemBioChem*, 4(8), pp.697–711.

Horvitz, H.R. et al., 1983. Mutations that affect neural cell lineages and cell fates during the development of the nematode Caenorhabditis elegans. *Cold Spring Harbor symposia on quantitative biology*, 48 Pt 2, pp.453–463.

Howlett, S.K. & Bolton, V.N., 1985. Sequence and regulation of morphological and molecular events during the first cell cycle of mouse embryogenesis. *Journal of embryology and experimental morphology*, 87, pp.175–206.

Hsu, D.C. & Prescr, C.K.A., 2009. Long-term management of patients taking immunosuppressive drugs. *Australian Prescriber*, 32, pp.68–71.

Hsu, T.-Y. & Wu, Y.-C., 2010. Engulfment of apoptotic cells in C. elegans is mediated by integrin alpha/SRC signaling. *Current biology : CB*, 20(6), pp.477–486.

Hu, C.Y. et al., 2009. Genetic polymorphism in milk fat globule-EGF factor 8 (MFG-E8) is associated with systemic lupus erythematosus in human. *Lupus*, 18(8), pp.676–681.

Huang, F.P. et al., 2000. A Discrete Subpopulation of Dendritic Cells Transports Apoptotic Intestinal Epithelial Cells to T Cell Areas of Mesenteric Lymph Nodes. *The Journal of Experimental Medicine*, 191(3), pp.435–444.

Huang, J. et al., 2009. Activation of antibacterial autophagy by NADPH oxidases. *Proceedings of the National Academy of Sciences of the United States of America*, 106(15), pp.6226–6231.

Huelgas Morales, G. & Greenstein, D., 2018. C. elegans germline cell death, live! A. D. Chisholm, ed., 14(7), pp.e1007425–3.

Huynh, M.-L.N. et al., 2005. Defective Apoptotic Cell Phagocytosis Attenuates Prostaglandin E 2and 15-Hydroxyeicosatetraenoic Acid in Severe Asthma Alveolar Macrophages. *American Journal of Respiratory and Critical Care Medicine*, 172(8), pp.972–979.

Jiang, H.-S. & Wu, Y.-C., 2014. LIN-3/EGF promotes the programmed cell death of specific cells in Caenorhabditis elegans by transcriptional activation of the pro-apoptotic gene egl-1. A. Gartner, ed. *PLOS Genetics*, 10(8), p.e1004513.

Jörg, L.M., 2018. *Identification of redundant lipid scramblases required for cell corpse clearance in C. elegans embryos*.

Kachur, T.M., Audhya, A. & Pilgrim, D.B., 2008. UNC-45 is required for NMY-2 contractile function in early embryonic polarity establishment and germline cellularization in C. elegans. *Developmental biology*, 314(2), pp.287–299.

Kaletta, T. & Hengartner, M.O., 2006. Finding function in novel targets: C. elegans as a model organism. *Nature Reviews Drug Discovery*, 5(5), pp.387–399.

Kang, J. et al., 2011. Essential roles of snap-29 in C. elegans. *Developmental biology*, 355(1), pp.77–88.

Kastan, M.B. et al., 1991. Participation of p53 protein in the cellular response to DNA damage. *Cancer research*, 51(23 Pt 1), pp.6304–6311.

Kerr, J.F., Wyllie, A.H. & Currie, A.R., 1972. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *British journal of cancer*, 26(4), pp.239–257.

Kim, J.-Y. et al., 2013. Noncanonical Autophagy Promotes the Visual Cycle. *Cell*, 154(2), pp.365–376.

Kinchen, J.M. & Ravichandran, K.S., 2010. Identification of two evolutionarily conserved genes regulating processing of engulfed apoptotic cells. *Nature*, 464(7289), pp.778–782.

Kinchen, J.M. & Ravichandran, K.S., 2008. Phagosome maturation: going through the acid test. *Nature Reviews Molecular Cell Biology*, 9(10), pp.781–795.

Kinchen, J.M. et al., 2005. Two pathways converge at CED-10 to mediate actin rearrangement and corpse removal in C. elegans. *Nature*, 434(7029), pp.93–99.

Kirisako, T. et al., 2000. The reversible modification regulates the membrane-binding state of Apg8/Aut7 essential for autophagy and the cytoplasm to vacuole targeting pathway. *The Journal of cell biology*, 151(2), pp.263–276.

Kneen, M. et al., 1998. Green fluorescent protein as a noninvasive intracellular pH indicator. *Biophysical journal*, 74(3), pp.1591–1599.

Koedel, U. et al., 2009. Apoptosis Is Essential for Neutrophil Functional Shutdown and Determines Tissue Damage in Experimental Pneumococcal Meningitis J. N. Weiser, ed. *PLoS Pathogens*, 5(5), pp.e1000461–13.

Kothakota, S. et al., 1997. Caspase-3-generated fragment of gelsolin: effector of morphological change in apoptosis. *Science (New York, N.Y.)*, 278(5336), pp.294–298.

Krieser, R.J. et al., 2002. Deoxyribonuclease IIα is required during the phagocytic phase of apoptosis and its loss causes perinatal lethality. *Cell death and differentiation*, 9(9), pp.956–962.

Kroemer, G. et al., 2013. Immunogenic Cell Death in Cancer Therapy. *Annual Review of Immunology*, 31(1), pp.51–72.

Ktistakis, N.T. & Tooze, S.A., 2016. Digesting the Expanding Mechanisms of Autophagy. *Trends in cell biology*, 26(8), pp.624–635.

Kurosaka, K. et al., 2003. Silent cleanup of very early apoptotic cells by macrophages. *The Journal of Immunology*, 171(9), pp.4672–4679.

Kusunoki, R. et al., 2012. Roles of milk fat globule-epidermal growth factor 8 in intestinal inflammation. *Digestion*, 85(2), pp.103–107.

Kutscher, L.M. & Shaham, S., 2017. Non-apoptotic cell death in animal development. 24(8), pp.1326–1336.

Kyrylkova, K. et al., 2012. Detection of Apoptosis by TUNEL Assay. In *Phagocytosis and Phagosomes*. Methods in Molecular Biology. Totowa, NJ: Humana Press, pp. 41–47.

Leist, M. et al., 1997. Intracellular adenosine triphosphate (ATP) concentration: a switch in the decision between apoptosis and necrosis. *The Journal of Experimental Medicine*, 185(8), pp.1481–1486.

Levin, I. et al., 2012. Effects of laser polar-body biopsy on embryo quality. *Fertility and sterility*, 97(5), pp.1085–1088.

Levin, R., Grinstein, S. & Canton, J., 2016. The life cycle of phagosomes: formation, maturation, and resolution. *Immunological reviews*, 273(1), pp.156–179.

Levine, A.J., 2019. Targeting Therapies for the p53 Protein in Cancer Treatments. *Annual Review of Cancer Biology*, 3(1), pp.21–34.

Levine, B., 2005. Autophagy in cell death: an innocent convict? *The Journal of clinical investigation*, 115(10), pp.2679–2688.

Li, B.-Z. et al., 2013. Identification of MFG-E8 as a novel therapeutic target for diseases. *Expert Opinion on Therapeutic Targets*, 17(11), pp.000–000.

Li, W. et al., 2009. C. elegans Rab GTPase activating protein TBC-2 promotes cell corpse degradation by regulating the small GTPase RAB-5. *Development (Cambridge, England)*, 136(14), pp.2445–2455.

Lim, C.-Y. & Zoncu, R., 2016. The lysosome as a command-and-control center for cellular metabolism. *The Journal of cell biology*, 214(6), pp.653–664.

Liu, Y. et al., 1999. Glucocorticoids promote nonphlogistic phagocytosis of apoptotic leukocytes. *The Journal of Immunology*, 162(6), pp.3639–3646.

Llambi, F. & Green, D.R., 2011. Apoptosis and oncogenesis: give and take in the BCL-2 family. *Current Opinion in Genetics & Development*, 21(1), pp.12–20.

Lu, N. et al., 2011. Three sorting nexins drive the degradation of apoptotic cells in response to PtdIns(3)P signaling. J. York, ed. *Molecular biology of the cell*, 22(3), pp.354–374.

Machin, G., 2009. Non-identical monozygotic twins, intermediate twin types, zygosity testing, and the non-random nature of monozygotic twinning: A review N. Shur, ed. *American Journal of Medical Genetics Part C: Seminars in Medical Genetics*, 151C(2), pp.110–127.

Maro, B. & Verlhac, M.-H., 2002. Polar body formation: new rules for asymmetric divisions. *Nature cell biology*, 4(12), pp.E281–3.

Martinez, J. et al., 2011. Microtubule-associated protein 1 light chain 3 alpha (LC3)-associated phagocytosis is required for the efficient clearance of dead cells. *Proceedings of the National Academy of Sciences of the United States of America*, 108(42), pp.17396–17401.

Martinez, J. et al., 2015. Molecular characterization of LC3-associated phagocytosis reveals distinct roles for Rubicon, NOX2 and autophagy proteins. *Nature cell biology*, 17(7), pp.893–906.

Martinez, J. et al., 2016. Noncanonical autophagy inhibits the autoinflammatory, lupus-like response to dying cells. *Nature*, 533(7601), pp.115–119.

Matwee, C., Betts, D.H. & King, W.A., 2000. Apoptosis in the early bovine embryo. *Zygote*, 8(1), pp.57–68.

McCarter, J.E.A., 1998. On the Control of Oocyte Meiotic Maturation and Ovulation in Caenorhabditis elegans. pp.1–18.

McEwan, D.G. et al., 2015. PLEKHM1 Regulates Autophagosome-Lysosome Fusion through HOPS Complex and LC3/GABARAP Proteins. *Molecular Cell*, 57(1), pp.39–54.

McNeil, P.L. et al., 1983. Acidification of phagosomes is initiated before lysosomal enzyme activity is detected. *The Journal of cell biology*, 97(3), pp.692–702.

MD, R.W.V. et al., 2002. Impaired Clearance of Apoptotic Cells From Cystic Fibrosis Airways. *CHEST*, 121(Supplement), p.89S.

Mello, C.C., 2007. Return to the RNAi World: Rethinking Gene Expression and Evolution (Nobel Lecture). *Angewandte Chemie International Edition*, 46(37), pp.6985–6994.

Mitter, S.K. et al., 2014. Dysregulated autophagy in the RPE is associated with increased susceptibility to oxidative stress and AMD. *Autophagy*, 10(11), pp.1989–2005.

Mony, V.K., Benjamin, S. & O'Rourke, E.J., 2016. A lysosome-centered view of nutrient homeostasis. *Autophagy*, 12(4), pp.619–631.

Morimoto, K., Janssen, W.J. & Terada, M., 2012. Defective efferocytosis by alveolar macrophages in IPF patients. *Respiratory Medicine*, 106(12), pp.1800–1803.

Muñoz, L.E. et al., 2010. The role of defective clearance of apoptotic cells in systemic autoimmunity. *Nature Publishing Group*, 6(5), pp.280–289.

Nijhawan, D., Honarpour, N. & Wang, X., 2000. Apoptosis in neural development and disease. *Annual review of neuroscience*, 23(1), pp.73–87.

Nikoletopoulou, V. et al., 2013. Crosstalk between apoptosis, necrosis and autophagy. *BBAMCR*, 1833(12), pp.3448–3459.

Nourshargh, S. & Alon, R., 2014. Leukocyte Migration into Inflamed Tissues. *Immunity*, 41(5), pp.694–707.

Obeid, M. et al., 2006. Calreticulin exposure dictates the immunogenicity of cancer cell death. *Nature Medicine*, 13(1), pp.54–61.

Ohsumi, Y., 2001. Molecular dissection of autophagy: two ubiquitin-like systems. *Nature Reviews Molecular Cell Biology*, 2(3), pp.211–216.

Oikonomou, V. et al., 2018. Autophagy and LAP in the Fight against Fungal Infections: Regulation and Therapeutics. *Mediators of inflammation*, 2018, pp.6195958–7.

Olson, S.K. et al., 2012. Hierarchical assembly of the eggshell and permeability barrier in C. elegans. *The Journal of cell biology*, 198(4), pp.731–748.

Opferman, J.T. & Korsmeyer, S.J., 2003. Apoptosis in the development and maintenance of the immune system. *Nature immunology*, 4(5), pp.410–415.

Otsuki, J. et al., 2012. Symmetrical division of mouse oocytes during meiotic maturation can lead to the development of twin embryos that amalgamate to form a chimeric hermaphrodite. *Human Reproduction*, 27(2), pp.380–387.

Otto, S.P., 2008. Sexual Reproduction and the Evolution of Sex. *Nature Education*, 1(1), p.182.

Ottolini, C.S. et al., 2015. Karyomapping identifies second polar body DNA persisting to the blastocyst stage: implications for embryo biopsy. *Reproductive BioMedicine Online*, 31(6), pp.776–782.

Ouyang, L. et al., 2012. Programmed cell death pathways in cancer: a review of apoptosis, autophagy and programmed necrosis. *Cell Proliferation*, 45(6), pp.487–498.

Parenti, G., Andria, G. & Ballabio, A., 2015. Lysosomal Storage Diseases: From Pathophysiology to Therapy. *Annual Review of Medicine*, 66(1), pp.471–486.

Park, D. et al., 2011. Continued clearance of apoptotic cells critically depends on the phagocyte Ucp2 protein. *Nature*, 477(7363), pp.220–224.

Pasparakis, M. & Vandenabeele, P., 2015. Necroptosis and its role in inflammation. *Nature*, 517(7534), pp.311–320.

Plachot, M., 2001. Chromosomal abnormalities in oocytes. *Molecular and cellular endocrinology*, 183 Suppl 1, pp.S59–63.

Pohl, C. & Bao, Z., 2010. Chiral forces organize left-right patterning in C. elegans by uncoupling midline and anteroposterior axis. *Developmental cell*, 19(3), pp.402–412.

Poon, I.K.H. et al., 2014. Apoptotic cell clearance: basic biology and therapeutic potential. *Nature reviews. Immunology*, 14(3), pp.166–180.

Praitis, V. et al., 2001. Creation of low-copy integrated transgenic lines in Caenorhabditis elegans. *Genetics*, 157(3), pp.1217–1226.

Proskuryakov, S.Y.A., Konoplyannikov, A.G. & Gabai, V.L., 2003. Necrosis: a specific form of programmed cell death? *Experimental Cell Research*, 283(1), pp.1–16.

Ravichandran, K.S. & Lorenz, U., 2007. Engulfment of apoptotic cells: signals for a good meal. *Nature reviews. Immunology*, 7(12), pp.964–974.

Reddien, P.W., Cameron, S. & Horvitz, H.R., 2001. Phagocytosis promotes programmed cell death in C. elegans. *Nature*, 412(6843), pp.198–202.

Riedl, J. et al., 2008. Lifeact: a versatile marker to visualize F-actin. *Nature Methods*, 5(7), pp.605–607.

Robertson, A.M.G. & Thomson, J.N., 1982. Morphology of programmed cell death in the ventral nerve cord of Caenorhabditis elegans larvae. *Development (Cambridge, England)*, 67(1), pp.89–100.

Rocheleau, C.E., 2018. C. elegans RAB-35: Dual roles in apoptotic cell clearance J. Nance, ed. *PLOS Genetics*, 14(8), pp.e1007534–4.

Rock, K.L. & Kono, H., 2008. The Inflammatory Response to Cell Death. *Annual Review of Pathology: Mechanisms of Disease*, 3(1), pp.99–126.

Rueda, B.R. et al., 1999. Accumulation of caspase-3 messenger ribonucleic acid and induction of caspase activity in the ovine corpus luteum following prostaglandin F2alpha treatment in vivo. *Biology of reproduction*, 60(5), pp.1087–1092.

Ruggiero, L. et al., 2012. Diurnal, localized exposure of phosphatidylserine by rod outer segment tips in wild-type but not Itgb5−/− or Mfge8−/− mouse retina. *Proceedings of the National Academy of Sciences*, 109(21), pp.8145–8148.

Rupp, J. et al., 2009. Chlamydia pneumoniae Hides inside Apoptotic Neutrophils to Silently Infect and Propagate in Macrophages D. M. Ojcius, ed. *PloS one*, 4(6), pp.e6020–8.

Sakahira, H., Enari, M. & Nagata, S., 1998. Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis. *Nature*, 391(6662), pp.96–99.

Sanjuan, M.A. et al., 2007. Toll-like receptor signalling in macrophages links the autophagy pathway to phagocytosis. *Nature*, 450(7173), pp.1253–1257.

Sarov, M. et al., 2012. A genome-scale resource for in vivo tag-based protein function exploration in C. elegans. *Cell*, 150(4), pp.855–866.

Sato, M. & Sato, K., 2011. Degradation of paternal mitochondria by fertilization-triggered autophagy in C. elegans embryos. *Science (New York, N.Y.)*, 334(6059), pp.1141–1144.

Sato, M. et al., 2005. Caenorhabditis elegans RME-6 is a novel regulator of RAB-5 at the clathrin-coated pit. *Nature cell biology*, 7(6), pp.559–569.

Savill, J.S. et al., 1989. Macrophage phagocytosis of aging neutrophils in inflammation. Programmed cell death in the neutrophil leads to its recognition by macrophages. *The Journal of clinical investigation*, 83(3), pp.865–875.

Schmerler, S. & Wessel, G.M., 2011. Polar bodies—more a lack of understanding than a lack of respect. *Molecular Reproduction and Development*, 78(1), pp.3–8.

Schrijvers, D.M. et al., 2005. Phagocytosis of Apoptotic Cells by Macrophages Is Impaired in Atherosclerosis. *Arteriosclerosis, Thrombosis, and Vascular Biology*, 25(6), pp.1256–1261.

Schultz, M.L. et al., 2011. Clarifying lysosomal storage diseases. *Trends in Neurosciences*, 34(8), pp.401–410.

Schulz-Schaeffer, J., 1980. Chromosomes During Sexual Reproduction. In *Cytogenetics*. New York, NY: Springer, New York, NY, pp. 128–146.

Schwartz, L.M. et al., 1993. Do all programmed cell deaths occur via apoptosis? *Proceedings of the National Academy of Sciences of the United States of America*, 90(3), pp.980–984.

Schwartz, S.L. et al., 2007. Rab GTPases at a glance. *Journal of cell science*, 120(Pt 22), pp.3905–3910.

Schweinsberg, P.J. & Grant, B.D., 2013. C. elegans gene transformation by microparticle bombardment. *WormBook : the online review of C. elegans biology*, pp.1–10.

Segawa, K. et al., 2014. Caspase-mediated cleavage of phospholipid flippase for apoptotic phosphatidylserine exposure. *Science (New York, N.Y.)*, 344(6188), pp.1164–1168.

Serhan, C.N. et al., 2007. Resolution of inflammation: state of the art, definitions and terms. *The FASEB Journal*, 21(2), pp.325–332.

Shaye, D.D. & Greenwald, I., 2011. OrthoList: a compendium of C. elegans genes with human orthologs. K. M. Iijima, ed. *PloS one*, 6(5), p.e20085.

Shen, Q. et al., 2013. Phagocytic receptor signaling regulates clathrin and epsin-mediated cytoskeletal remodeling during apoptotic cell engulfment in C. elegans. *Development (Cambridge, England)*, 140(15), pp.3230–3243.

Shi, A. et al., 2009. Regulation of endosomal clathrin and retromer-mediated endosome to Golgi retrograde transport by the J-domain protein RME-8. *The EMBO Journal*, 28(21), pp.3290–3302.

Shimomura, O., 2009. Discovery of Green Fluorescent Protein (GFP) (Nobel Lecture). *Angewandte Chemie International Edition*, 48(31), pp.5590–5602.

Singh, D. & Pohl, C., 2014. Coupling of rotational cortical flow, asymmetric midbody positioning, and spindle rotation mediates dorsoventral axis formation in C. elegans. *Developmental cell*, 28(3), pp.253–267.

Slee, E.A., Adrain, C. & Martin, S.J., 2001. Executioner Caspase-3, -6, and -7 Perform Distinct, Non-redundant Roles during the Demolition Phase of Apoptosis. *The Journal of biological chemistry*, 276(10), pp.7320–7326.

Sprenkeler, E.G.G., Gresnigt, M.S. & van de Veerdonk, F.L., 2016. LC3-associated phagocytosis: a crucial mechanism for antifungal host defence against Aspergillus fumigatus N. Gow & E. Hartland, eds. *Cellular Microbiology*, 18(9), pp.1208–1216.

Srayko, M. et al., 2000. MEI-1/MEI-2 katanin-like microtubule severing activity is required for Caenorhabditis elegans meiosis. *Genes & Development*, 14(9), pp.1072–1084.

Stenmark, H. & Olkkonen, V.M., 2001. The Rab GTPase family. *Genome biology*, 2(5), p.REVIEWS3007.

Stuart, L.M. et al., 2005. Mannose-Binding Lectin-Deficient Mice Display Defective Apoptotic Cell Clearance but No Autoimmune Phenotype. *The Journal of Immunology*, 174(6), pp.3220–3226.

Suh, C.-I. et al., 2006. The phosphoinositide-binding protein p40phox activates the NADPH oxidase during FcγIIA receptor–induced phagocytosis. *The Journal of Experimental Medicine*, 203(8), pp.1915–1925.

Sulston, J.E., 2003. Caenorhabditis elegans: The Cell Lineage and Beyond (Nobel Lecture). *ChemBioChem*, 4(8), pp.688–696.

Sulston, J.E., 1976. Post-Embryonic Development in the Ventral Cord of Caenorhabditis elegans. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 275(938), pp.287–297.

Sulston, J.E. et al., 1983. The embryonic cell lineage of the nematode Caenorhabditis elegans. *Developmental biology*, 100(1), pp.64–119.

Suzuki, J., Imanishi, E. & Nagata, S., 2016. Xkr8 phospholipid scrambling complex in apoptotic phosphatidylserine exposure. *Proceedings of the National Academy of Sciences of the United States of America*, 113(34), pp.9509–9514.

Tabas, I., 2009. Macrophage death and defective inflammation resolution in atherosclerosis. *Nature reviews. Immunology*, 10(1), pp.36–46.

Tian, Y. et al., 2010. C. elegans Screen Identifies Autophagy Genes Specific to Multicellular Organisms. *Cell*, 141(6), pp.1042–1055.

Trapani, J.A. & Smyth, M.J., 2002. Functional significance of the perforin/granzyme cell death pathway. *Nature reviews. Immunology*, 2(10), pp.735–747.

Tsien, R.Y., 2010. Nobel lecture: constructing and exploiting the fluorescent protein paintbox. *Integrative biology : quantitative biosciences from nano to macro*, 2(2-3), pp.77–93.

Tsuboyama, K. et al., 2016. The ATG conjugation systems are important for degradation of the inner autophagosomal membrane. *Science (New York, N.Y.)*, 354(6315), pp.1036–1041.

Tsujimoto, Y. & Shimizu, S., 2005. Another way to die: autophagic programmed cell death. *Cell death and differentiation*, 12(S2), pp.1528–1534.

Verges, M., 2007. Retromer and sorting nexins in development. *Frontiers in bioscience : a journal and virtual library*, 12, pp.3825–3851.

Verlinsky, Y. et al., 1997. Prepregnancy genetic testing for age-related aneuploidies by polar body analysis. *Genetic testing*, 1(4), pp.231–235.

Vlachos, M. & Tavernarakis, N., 2010. Non-apoptotic cell death in Caenorhabditis elegans. M. Han, ed. *Developmental dynamics : an official publication of the American Association of Anatomists*, 239(5), pp.1337–1351.

Wallen, C.A., Higashikubo, R. & Dethlefsen, L.A., 1982. Comparison of two flow cytometric assays for cellular RNA--acridine orange and propidium iodide. *Cytometry*, 3(3), pp.155–160.

Wang, C.-W. & Klionsky, D.J., 2003. The molecular mechanism of autophagy. *Molecular medicine (Cambridge, Mass.)*, 9(3-4), pp.65–76.

Wang, X., 2003. Cell Corpse Engulfment Mediated by C. elegans Phosphatidylserine Receptor Through CED-5 and CED-12. *Science (New York, N.Y.)*, 302(5650), pp.1563–1566.

Wang, X. & Yang, C., 2016. Programmed cell death and clearance of cell corpses in Caenorhabditis elegans. *Cellular and Molecular Life Sciences*, 73(11), pp.2221–2236.

Wang, X. et al., 2007. C. elegans mitochondrial factor WAH-1 promotes phosphatidylserine externalization in apoptotic cells through phospholipid scramblase SCRM-1. *Nature cell biology*, 9(5), pp.541–549.

Wang, X. et al., 2010. Caenorhabditis elegans transthyretin-like protein TTR-52 mediates recognition of apoptotic cells by the CED-1 phagocyte receptor. 12(7), pp.655–664.

Wang, X. et al., 2002. Mechanisms of AIF-mediated apoptotic DNA degradation in Caenorhabditis elegans. *Science (New York, N.Y.)*, 298(5598), pp.1587–1592.

Wehman, A.M. et al., 2011. The P4-ATPase TAT-5 inhibits the budding of extracellular vesicles in C. elegans embryos. *Current biology : CB*, 21(23), pp.1951–1959.

Wu, Y.C. et al., 2001. C. elegans CED-12 acts in the conserved crkII/DOCK180/Rac pathway to control cell migration and cell corpse engulfment. *Developmental cell*, 1(4), pp.491–502.

Wu, Y.C., Stanfield, G.M. & Horvitz, H.R., 2000. NUC-1, a caenorhabditis elegans DNase II homolog, functions in an intermediate step of DNA degradation during apoptosis. *Genes & Development*, 14(5), pp.536–548.

Yamaguchi, H. et al., 2010. Aberrant splicing of the milk fat globule-EGF factor 8 (MFG-E8) gene in human systemic lupus erythematosus. *European Journal of Immunology*, 40(6), pp.1778–1785.

Yang, H. et al., 2014. A lysine-rich motif in the phosphatidylserine receptor PSR-1 mediates recognition and removal of apoptotic cells. *Nature Communications*, 6, pp.1–12.

Yang, P. & Zhang, H., 2011. The coiled-coil domain protein EPG-8 plays an essential role in the autophagy pathway in C. elegans. *Autophagy*, 7(2), pp.159–165.

Yu, X., Lu, N. & Zhou, Z., 2008. Phagocytic receptor CED-1 initiates a signaling pathway for degrading engulfed apoptotic cells. K. L. Simons, ed. *PLoS Biology*, 6(3), p.e61.

Zakeri, Z. et al., 2005. A generalized caspase inhibitor disrupts early mammalian development. *The International Journal of Developmental Biology*, 49(1), pp.43–51.

Zeiss, C.J., 2003. The Apoptosis-Necrosis Continuum: Insights from Genetically Altered Mice. *Veterinary pathology*, 40(5), pp.481–495.

Zhou, Z. et al., 2001. The C. elegans PH domain protein CED-12 regulates cytoskeletal reorganization via a Rho/Rac GTPase signaling pathway. *Developmental cell*, 1(4), pp.477–489.