Regulation of autophagy-related mechanisms during bacterial infection

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Chapter 6

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Summary and discussion

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Autophagy is a fundamental degradative process, important for maintaining cellular homeostasis. It captures dysfunctional cellular components such as misfolded proteins or defective organelles into a double membrane autophagosome and delivers them to lysosomes for degradation (Parzych and Klionsky, 2014). Autophagy is also regarded as a crucial part of the innate immune system (Deretic and Levine, 2009) (**Chapter 1**). Antibacterial autophagy, also as known as xenophagy, functions in the host defense against intracellular pathogens, such as *Mycobacterium tuberculosis* (Mtb), *Salmonella enterica*, *Listeria monocytogenes*, *Shigella flexneri*, and many others (Deretic and Levine, 2009; Huang and Brumell, 2014). Normally, during infection, such pathogens are initially internalized by a host cell into vesicles of phagosomal origin. This phagocytosis process is the default defense mechanism to deliver pathogens to lysosomes for degradation. However, several intracellular pathogens can survive within the host cell because they arrest the fusion of phagosomes with lysosomes, and thus avoid the microbicidal mechanisms of these organelles. In addition, these pathogens often cause damage to the membrane of the phagosome and thus gain access to the cytosol. The cytosolic bacteria can then be recognized by the autophagy machinery, providing a second line of defense to direct such pathogens to lysosomes for degradation (Deretic et al., 2006). Obviously, successful pathogens must be equipped with virulence factors to defend themselves against autophagy.

Autophagy is regulated by a variety of proteins. Damage Regulated Autophagy Modulator 1 (DRAM1) is one of these regulators, induced by stress and infection. It was first found that DRAM1 mediates autophagy and induces cell death after induction by transcription factor p53 (Crighton et al., 2006). In our laboratory, utilizing zebrafish and human macrophages as model systems, it was found that DRAM1 is induced after mycobacterial infection by the components of Toll-like receptor (TLR) signaling pathway, including the TLR-adaptor MYD88 and transcription factor NF-κB (van der Vaart et al., 2014). Further studies on the zebrafish homologue of DRAM1 (Dram1) demonstrated its role in autophagic defense against mycobacterial infection (van der Vaart et al., 2014). Generation of Dram1-deficient zebrafish confirmed that Dram1 is required to restrict the proliferation of mycobacteria inside macrophages, because the absence of Dram1 impairs the autophagic and lysosomal responses to the pathogen, and leads to an increased level of infected cell death (Zhang et al., 2020). However, it remained to be clarified

how DRAM1 augments autophagy to kill bacteria. Therefore, the aim of the studies in this thesis was to unravel the bactericidal mechanisms underlying the function of DRAM1.

DRAM1 promotes LC3-associated phagocytosis in *Salmonella* infection

Like Mtb, *Salmonella* bacteria can reside intracellularly in host cells, including phagocytic cells like macrophages. After phagocytosis, *Salmonella* stays in a modified vesicle called the *Salmonella*-containing vacuole (SCV). However, *Salmonella* can apply effector molecules of its type III secretion systems (T3SSs) to cause membrane damage to SCVs. T3SSs are needle-like structures located on the bacterial cell wall. These sharp structures can penetrate the cellular or intracellular membranes of host cells and form pores. Due to this pore-forming activity, the bacteria can escape from the SCVs and enter the cytosol, which triggers xenophagy as a defense response (Wu et al., 2020). In addition, the autophagy machinery can also target *Salmonella* when it is still inside the phagosome or SCV. This non-canonical autophagy pathway is known as LC3-associated phagocytosis (LAP) (Masud et al., 2019).

LAP is initiated following the recognition of pathogens by TLRs (Sanjuan et al., 2007). The hallmark of LAP is the conjugation of the autophagy protein LC3 to the membrane of the phagosome (Sanjuan et al., 2007). This step requires phosphatidylinositol 3-phosphate (PI3P) generation by type III phosphatidylinositol 3-kinase complex (PI3KC3), which consists of Beclin1, VPS34, VPS15, UVRAG, and Rubicon (Martinez et al., 2015). PI3P is dispersed on the phagosomal membrane and recruits the p40phox subunit for assembly of the multiprotein NADPH oxidase complex (Bagaitkar et al., 2017). Rubicon stabilizes another NADPH oxidase subunit, p22phox (Martinez et al., 2015). When all the subunits are recruited and assembled, NADPH oxidase produces ROS inside the lumen of the phagosome. ROS production by NADPH oxidase consumes H^+ and results in a higher pH in the phagosomes, which induces V-ATPase assembly. The V-ATPase interacts with the autophagy protein ATG16L1 through its WD40 domain and thereby drives the formation on the ATG12–ATG5–ATG16L complex mediating LC3 conjugation to the phagosome, resulting in a LAPosome (Hooper et al., 2022). A notable difference between autophagy and LAP is that LC3 is conjugated to the double membranes of autophagosomes and to the single membranes of LAPosomes.

Previous work has shown that deficiency of Rubicon, NADPH oxidase subunit Cyba, and Atg5, reduced the colocalization of Lc3 with bacteria and the ROS response (Masud et al., 2019). However, deficiency of Atg13, which is a component of the Ulk1 complex exclusively required for autophagy

initiation, showed no difference in the Lc3 and ROS responses. This suggests that LAP rather than xenophagy is the main autophagy-related pathway to restrict growth of *Salmonella enterica* serovar *Typhimurium* (ST) within macrophages of systemically infected zebrafish embryos (Masud et al., 2019). We therefore employed the zebrafish-ST infection model to investigate the possible role of DRAM1 in LAP (**Chapter 2**). To confirm the role of LAP in ST infection, we used a drug, 2-(tetrahydroindazolyl) phenoxy-N-(thiadiazolyl) propenamide (TIPTP), to inhibit the interaction of Rubicon and NADPH oxidase. We assessed the ROS production associated with LAP using a *Salmonella* ROS biosensor strain, and found that ROS production was decreased with TIPTP treatment, both in zebrafish embryos and in a mouse macrophage cell line (RAW 264.7). These results of chemical inhibition are in line with previous knockdown studies of Rubicon and NADPH oxidase activity (Kim et al., 2020), thus strengthening the evidence that LAP is a conserved defense mechanism against ST infection. Furthermore, we found that zebrafish embryos with Dram1 deficiency failed to restrict ST growth and consequently increased mortality was observed, while overexpression of Dram1 reduced mortality. Furthermore, we found that DRAM1/Dram1 deficiency decreased ROS production and colocalization of LC3/Lc3 with ST in both RAW 264.7 macrophages and zebrafish, while overexpression augmented these responses. These results let us to conclude that DRAM1 plays a host protective role against ST infection through stimulation of the LAP pathway (**Chapter 2**).

The role of DRAM1 in LAP could be due to its proposed stimulatory effect on a late step in LAP, namely the fusion between bacteria-containing vesicles and lysosomes, similar to its role in the autophagy pathway (van der Vaart et al., 2014; Zhang et al., 2020) (**Chapter 4 and 5)**. This effect could be mediated by the interaction of DRAM1 with SNARE protein VTI1B **(chapter 5)**. However, our results obtained with the ROS biosensor strain position DRAM1 as a mediator of the LAP-associated ROS response, raising the question how DRAM1 might mediate both early and late steps in the LAP pathway (**Fig.1**). A previous study reported that DRAM1 is required to recruit the V-ATPase V1 subunit, and therefore promotes the assembly of the V-ATPase complex (Zhang et al., 2013). The V-ATPase complex functions in lysosome acidification, but has recently also been implicated in LAP, functioning upstream of LC3 conjugation (Hooper et al., 2022). Furthermore, the V-ATPase V0 subunit has been found to participate in different membrane fusions (Marshansky and Futai, 2008). Thus, an effect of DRAM1 on V-ATPase activity would be consistent with both early and late roles of DRAM1 in LAP. However, there is currently no evidence that DRAM1 is physically interacting

with V-ATPase components (Geng et al., 2020). Another possibility is that DRAM1 interacts with a component of the PI3KC3 complex, as it is known that generation of PI3P by PI3KC3 is a prerequisite for ROS production during LAP. DRAM2, another member of the DRAM family, has been shown to interact with BECN1 and UVRAG to increase PI3KC3 activity (Kim et al., 2017). We therefore hypothesize that DRAM1 has a similar function to increase PI3KC3 activity, thus promoting PI3P generation, ROS production, and subsequent LC3 conjugation.

Knowing that DRAM1 functions in LAP as well as in autophagy, the question arises if DRAM1 activity can switch between these two pathways or affect both of them simultaneously. UVRAG regulates PI3KC3 activity during autophagosome and endosome maturation. However, Rubicon sequesters UVRAG's function to inhibit autophagosome and endosome maturation (Sun et al., 2010). By analogy with what has been proposed for DRAM2, we hypothesize that DRAM1 is able to release Rubicon from the PI3KC3 complex (Kim et al., 2017). DRAM1/DRAM2-mediated displacement of Rubicon would relieve its inhibition and thus promote autophagosome maturation. At the same time, Rubicon protein liberated from the PI3KC3 complex may be recruited to stabilize the phagosomal NADPH oxidase, thus driving the ROS production required for LAP. This hypothesis would explain how DRAM1 could stimulate autophagy and LAP at the same time.

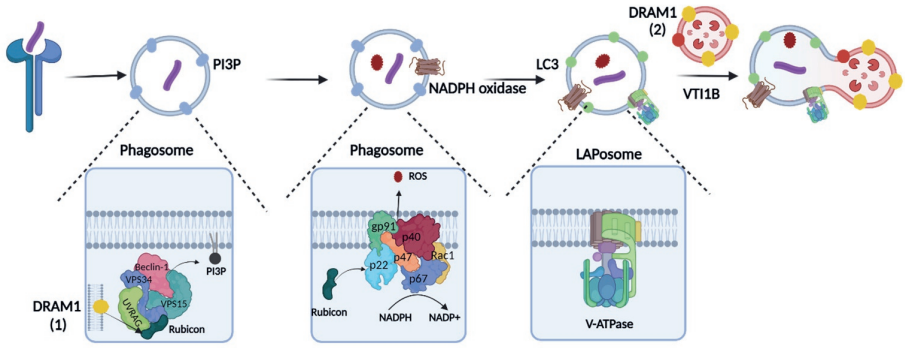

Figure 1: Proposed roles of DRAM1 in the LAP pathway. Studies on the role of DRAM1 in LAP-mediated defense against *Salmonella* indicate that DRAM1 promotes a step upstream of the ROS production that is required for LC3 recruitment to the phagosome, generating the LAPosome (**Chapter 3**). Potentially, this effect is mediated by an interaction between DRAM1 and UVRAG in the PI3KC3 complex (1), thereby generating PI3P and activating Rubicon, which stabilizes NADPH oxidase to produce ROS. ROS subsequently activate the V-ATPase, which initiates LC3 conjugation through interaction with ATG16L. Additionally, downstream of ROS production, the interaction between DRAM1 and SNARE protein VTI1B (2) could promote the fusion of LAPosomes with lysosomes (**Chapter 5**).

Dram1 can promote zebrafish host defense against *Mycobacterium marinum* independently of xenophagy receptors p62 and Optn

The initiation of antibacterial autophagy by the xenophagy pathway depends on recognition of pathogens by selective autophagy receptors, such as optineurin (Optn) or sequestosome 1 (p62) (Sharma et al., 2018). *Mycobacterium marinum* (Mm) is a frequently used model for studying the pathogenesis of tuberculosis. Mm shares the majority of its virulence factors with the human tuberculosis pathogen, Mtb. Both Mm and Mtb use the 6 kDa early secretory antigenic target (ESAT6) protein family secretion (ESX) 1 system to rupture phagosomal membranes, which enables these mycobacteria to enter the cytosol of macrophages (van der Wel et al., 2007). By using the zebrafish-Mm infection model, previous studies in our laboratory showed that the host defense against Mm is impaired by deficiencies in p62 or Optn (Zhang et al., 2019). Here, we have further investigated the relationship between these two receptors and their interaction with Dram1 in the zebrafish host defense against Mm (**Chapter 3**). By overexpressing mRNA in previously established CRISPR/Cas9 knockout zebrafish lines, we showed that Optn and p62 can compensate for the loss of each other's function, since overexpression of a single receptor was found to restore the infection susceptibility of the other mutant phenotype. Subsequently, by generating an *optn*/*p62* double mutant zebrafish line, we could show the additive effects of Optn and p62 in controlling bacterial burden, since the double mutant zebrafish line showed higher bacterial burden compared the single mutant. Thus, we concluded that the two receptors, Optn and p62, do not rely on each other for their role in the defense against Mm infection in zebrafish. Instead, both of them contribute and each of them can promote host defense independently.

In contrast to our study of mycobacterial infection, no additive effect on *Salmonella* proliferation in HeLa cells was found when double knockdown of *OPTN* was performed with either *p62* or another autophagy receptor, *NDP52*, suggesting an interdependency between these receptors in the xenophagy pathway against *Salmonella* (Wild et al., 2011). It is possible that the interaction between OPTN and p62 is pathogen-specific. Alternatively, in the context of a whole organism, mutation or overexpression of the receptors might have effects beyond xenophagy. For example, inflammation in the zebrafish host might be affected, as selective autophagy receptors are known to interact with several proteins in inflammatory signaling pathways (Oakes et al., 2017). Finally, the knockdown models or mutants could differ in the

expression levels of other selective autophagy receptors, which may be modulated as a compensatory mechanism. Indeed, our *optn*/*p62* double mutant zebrafish embryos showed increased *ndp52* expression.

Following up on earlier work establishing that Dram1 plays an important role in defense against Mm infection in zebrafish (van der Vaart et al., 2014; Zhang et al., 2020), we asked if Dram1 would still be able to function properly when selective autophagy is impaired (**Chapter 3**). We found that *dram1* overexpression can compensate for single or double deficiencies in Optn and p62, indicating that Dram1 does not rely on these receptors for its role in the host defense against Mm. Besides reducing the bacterial burden, *dram1* overexpression in *optn/p62* double mutants increased Lc3 colocalization with Mm. Thus, in the absence of *optn* and *p62*, other selective autophagy receptors, including Ndp52 whose expression level was shown to be increased, may capture Mm into autophagosomes and deliver the bacteria to lysosomes in a Dram1-dependent manner. In RAW 264.7 macrophages, we showed that DRAM1 promotes vesicle fusions mediating autophagosome maturation and antimicrobial peptide delivery (**Chapter 4,5**). It is likely that these Dram1-dependent mechanisms are also functional in the zebrafish model. Another possibility is that Dram1 promotes defense against Mm through the autophagy-related LAP pathway as previously shown for ST (**Chapter 2**).

While *dram1* overexpression improved the resistance of *optn*/*p62* double mutant zebrafish to Mm infection, overexpression of *optn* or *p62* could, vice versa, improve the host resistance of *dram1* mutant zebrafish embryos (**Chapter 3**). Our results in RAW 264.7 macrophages (**Chapter 5**) provide a possible explanation for why overexpression of *Optn* and *p62* can increase host resistance even in the absence of Dram1. Based on these results we believe that Dram1 plays a stimulatory but dispensable role in vesicle fusions through interaction with the SNARE protein VTI1B (**Chapter 5**). Thus, increasing selective autophagy of Mm in *dram1* mutant background, can still result in lysosomal delivery of Mm in a Dram1-independent manner. Taken together, the results suggest that Optn, p62, and Dram1 are all critical, yet limiting factors for restraining Mm proliferation in the zebrafish model. Furthermore, our results indicate the presence of compensatory mechanisms in the zebrafish innate immune response, where selective autophagy receptors and Dram1 can independently boost the antibacterial defenses.

DRAM1 promotes and lysosomal fusion and antimicrobial peptide delivery

To gain a better understanding of the molecular and cellular functions of DRAM1, we utilized RAW 264.7 macrophages as a cellular infection model to build further on the previous results of Mm infection in the zebrafish model (van der Vaart et al., 2014; Zhang et al., 2020). By immunostaining we found that the DRAM1 localization pattern gradually progressed from a punctate pattern to a full envelopment of the Mm bacteria (**Chapter 4**). These staining patterns were concomitant with colocalization of the autophagy marker LC3, the lysosomal marker LAMP1, and the LysoTracker dye for acidic vesicles, which strengthened our hypothesis that DRAM1 functions in trafficking of mycobacteria along the (auto)phagolysosomal pathway. To further test this hypothesis, we generated *Dram1* knockdown RAW 264.7 macrophage lines by lentiviral-mediated shRNA knockdown. As expected, we found that DRAM1 is required for autophagy-mediated defense against Mm, since DRAM1 knockdown led to reduced LC3 colocalization with Mm. In addition, we found that acidification of Mm-containing vesicles was impaired, as LysoTracker and LAMP1 colocalization with Mm were decreased by DRAM1 deficiency. To confirm the host defense function of DRAM1, we showed that DRAM1 deficiency led to higher infection rates and induced more infected cell death (**Chapter 4**).

Next, we explored antimicrobial peptide delivery as a possible mechanism by which DRAM1 could mediate the killing of pathogens in an autophagy-dependent manner (**Chapter 5**). Previous studies showed that the cytosolic protein Fau, a ubiquitin-like protein fused to the ribosomal S30 subunit, is captured by autophagosomes. After fusion with lysosomes, Fau is processed into neo-antimicrobial peptides and delivered into mycobacteria-containing vesicles (Ponpuak et al., 2010). To study if DRAM1 is involved in Fau delivery, we utilized the wildtype (WT) and a virulence-factor-deficient strain of Mm known as ΔRD1 (Region of difference 1). The RD1 locus encodes the ESX-1 system along with its secreted proteins, ESAT-6 and CFP-10 (Smith et al., 2008). ESAT-6 is a pore-forming protein that disrupts the integrity of the phagosomal membrane, thereby arresting the fusion of phagosomes and lysosomes (Gröschel et al., 2016). Indeed, we found that in infected RAW 264.7 macrophages, WT Mm showed more colocalization with galectin-3, a marker for damaged membranes, compared to ΔRD1 Mm. In agreement, WT Mm colocalized more frequently with ubiquitin, which is an indicator of cytosolic residence. Fau, on the other hand, colocalized more frequently with ΔRD1 Mm, supporting that Fau reaches bacteria that are inside vesicles and that it can be considered a faithful marker for antimicrobial peptide delivery.

We subsequently found that endosomal markers (Rab5 and Rab7), as well as lysosomal markers (LAMP1 and LysoTracker), colocalized more frequently with ΔRD1 Mm, indicating that vesicle fusions associated with the acquisition of these markers could drive Fau delivery to these bacteria-containing vesicles. It is possible that Fau-containing autophagosomes fuse with these bacteria-containing vesicles (Peña-Ramos et al., 2022), or that, in the case of cytosolic invasion, Fau is delivered by xenophagy (**Fig. 2**).

To understand if DRAM1 is involved in the Fau delivery process, we utilized *Dram1* knockdown RAW 264.7 macrophage cell lines. *Dram1* knockdown leads to decreased colocalization of both WT and ΔRD1 Mm with LC3 (**Chapter 4, 5**), consistent with previous studies (Lu et al., 2019; van der Vaart et al., 2014; Zhang et al., 2020). This indicates that fewer autophagosomes are formed. Concomitantly, the capturing of Fau in Mm-containing autophagosomes was impaired, as indicated by reduced Fau and LC3 colocalization in *Dram1* knockdown cells (**Chapter 5**). While DRAM1 has been shown to promote autophagosome-lysosome fusion (Zhang et al., 2013), other studies suggested that it is also involved in autophagosome formation (Lu et al., 2019; van der Vaart et al., 2014; Zhang et al., 2020), which could explain the effect of DRAM1 knockdown on Fau and LC3 colocalization.

However, it has never really been clarified how DRAM1 promotes autophagosome formation. A possible explanation might be found in the observation that DRAM1 localizes not only to autophagosomes and lysosomes but also to the plasma membrane (**Chapter 4**). Clathrin-mediated endocytosis provides plasma membrane for phagophore formation, which precedes the formation of a complete autophagosome. This phagophore formation is dependent on a SNARE protein complex including VTI1B (Moreau et al., 2011). In our study, we found that DRAM1 physically interacts with VTI1B (**Chapter 5**). We hypothesize that the interaction of DRAM1 with VTI1B activates the SNARE complex to initiate autophagy by phagophore formation. This provides one explanation why DRAM1 deficiency results in reduced autophagosome formation and consequently less Fau delivery to bacteria inside autophagy-derived vesicles.

The interaction between DRAM1 and VTI1B could also promote vesicle fusions in the autophagy pathway or during phagocytosis, which provides another mechanistic explanation for how DRAM1 could promote Fau delivery (**Fig.2**). After completing the formation of autophagosomes, they undergo stepwise maturation steps. Autophagosomes may fuse with endosomes and multivesicular bodies to form amphisomes, which subsequently fuse with lysosomes (Ganesan and Cai, 2021). Alternatively, autophagosomes fuse directly with lysosomes, forming autolysosomes (Zhao et al., 2021). In our

study, we found that DRAM1 is required for Mm acidification, as DRAM1 deficiency reduced colocalization of Mm with LAMP1 or LysoTracker (**Chapter 4, 5**). These results support the idea that that DRAM1 affects the trafficking of acidic vesicles to Mm-containing vesicles. Such a role of DRAM1 in promoting acidic vesicle fusion with Mm-containing compartments was suggested earlier, based on results in zebrafish overexpressing *dram1*, where large composite vesicles containing bacteria together with remnants from vesicle membranes were observed in transmission electron micrographs (van der Vaart et al., 2014). To understand in which fusion steps DRAM1 is involved, we performed double staining of the autophagosome marker LC3 and different vesicle markers, indicating that DRAM1 promotes autophagosome fusion with multiple vesicle types, including early endosomes, late endosomes, multivesicular bodies, and lysosomes (**Fig. 2**). In agreement, DRAM1 knockdown affected Fau delivery to all these types of Mm containing vesicles (**Chapter 5**).

As discussed above, we identified the SNARE protein VTI1B as a DRAM1 interaction partner. Besides functioning in plasma membrane-derived phagophore formation, VTI1B has been shown to promote autophagosome-lysosome fusion during antibacterial autophagy (Furuta et al., 2010). We found that VTI1B colocalization with Mm is decreased by DRAM1 deficiency (**Chapter 5**). We therefore propose that the interaction between DRAM1 and VTI1B promotes vesicle fusions underlying autophagosome maturation into autolysosomes, which would increase the processing of Fau into antimicrobial peptides due to the progressively acidic environment (**Fig.2**). Taken together, DRAM1 is believed to increase both phagophore formation and autophagosome maturation to deliver Fau to Mm-containing vesicles and enhance the antibacterial properties of these vesicles (**Chapter 5**).

Conclusion

The results of this thesis have increased insight into the function of an important regulator of antibacterial autophagy, DRAM1, which protects against infection with both mycobacteria and *Salmonella*. DRAM1 restricts bacterial growth not only through canonical antibacterial autophagy (xenophagy) but also promotes an autophagy-related pathway, named LC3-associated phagocytosis (**Chapter 2**). The function of DRAM1 in restricting bacterial proliferation is independent from the recognition of bacteria by xenophagy receptors (**Chapter 3**). Mechanistically, DRAM1 promotes the infection-induced activation of autophagy and LAP as well as the maturation of bacteria-containing vesicles in both pathways (**Chapter 3, 4, 5**). This

maturation process, stimulated by DRAM1, involves multiple vesicle fusion steps directing bacteria to lysosomes (**Chapter 4,5**). Through this maturation process, DRAM1 delivers the cytosolic protein Fau to bacteria-containing vesicles, where it serves as a precursor for antimicrobial peptides (**Chapter 5**). The underlying mechanism may be explained by the discovery of an interaction between DRAM1 and the SNARE protein VTI1B (**Chapter 5**). This finding encourages us to further study the crosstalk between DRAM1 and SNARE proteins in autophagy and LAP. Overall, the work in this thesis contributes to ongoing research into the potential application of autophagy modulation as a host-directed therapy against infectious diseases.

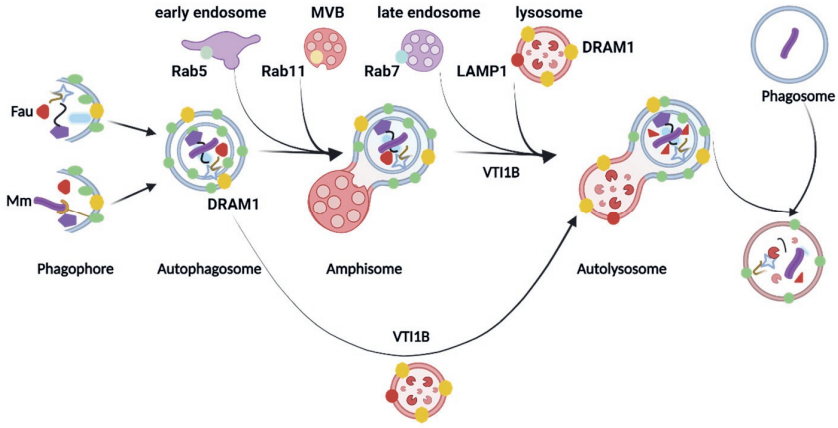

Figure 2: Schematic representation of DRAM1-mediated vesicle maturation step facilitating cytosolic Fau delivery to intravesicular bacteria. Autophagy activation captures the cytosolic protein Fau or cytosolic bacteria into an autophagosome, which undergoes a stepwise maturation process promoted by DRAM1. The autophagosome fuses with early endosomes or multivesicular bodies (MVB) to become an amphisome, which then fuses with late endosomes and lysosomes. Alternatively, the autophagosome fuses directly with lysosomes to form autolysosomes. DRAM1 interaction with VTI1B (**Chapter 5**) is proposed to mediate this step. Autolysosomes may also fuse with phagosomes as an alternative route of delivering Fau to bacteria-containing vesicles.

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