Dmon1 controls recruitment of Rab7 to maturing endosomes in Drosophila

Jahan Yousefian, Tobias Troost, Ferdi Grawe, Takeshi Sasamura*, Mark Fortini† and Thomas Klein‡

Institut für Genetik, Heinrich-Heine-Universität Düsseldorf, Universitätsstrasse 1, 40225 Düsseldorf, Germany
*Present address: Department of Biochemistry and Molecular Biology, Thomas Jefferson University, BLSB 830, 233 South 10th Street, Philadelphia, PA 19107, USA
†Author for correspondence (thomas.klein@uni-duesseldorf.de)

Summary
The small GTPases Rab5 and Rab7 are important organisers of endosome formation and maturation. In addition, they orchestrate the trafficking of cargo through the endosomal pathway. A crucial event during maturation of endosomes is the replacement of the early organiser Rab5 with the late organiser Rab7 in a process called Rab conversion. Rab conversion is a prerequisite for late events, chief among them the fusion of matured endosomes with the lysosome. Recent work identifies members of the Sand1/Mon1 protein family as crucial factors during this process. Here, we present an analysis of the function of the Drosophila ortholog of mon1/sand1, Dmon1. We found that loss of function of Dmon1 results in an enlargement of maturing endosomes and loss of their association with Rab7. The enlarged endosomes contain Notch and other trans-membrane proteins as cargo. We report the first electron microscopy analysis of Dmon1 cells in a metazoan and extend the analysis of the endosomes in mutant cells. Our results suggest that the phenotype can be explained by the loss of function of Rab7. Moreover, the endosomes of Dmon1 cells mature normally in many aspects, despite the loss of association with Rab7. Surprisingly, we did not observe overactive or ectopic signalling through receptors such as Notch and RTKs in Dmon1 mutant cells, as would have been expected because of the accumulation of receptors in the maturing endosomes of these cells. This was the case even when receptor uptake into intraluminal vesicles was suppressed.

Key words: Mon1, Notch, Rab7, Sand1, Cell signaling, endosomal trafficking

Introduction
In eukaryotic cells a highly dynamic endomembrane system is responsible for the compartmentalisation and maintenance of several organelles as well as the transport of cargo between them (reviewed by Huotari and Helenius, 2011). During endosomal trafficking, transmembrane proteins destined for degradation are incorporated in small endocytic vesicles (EV) that have pinched off the plasma membrane during endocytosis. These EVs fuse to form the early endosome (EE). From the EE, receptors can relocate to the plasma membrane through Rab4-controlled fast or Rab11-controlled slow recycling pathways. Cargo destined for degradation remains in the maturing endosome (ME), which eventually fuses with the lysosome, where its cargo is degraded. During maturation of the endosome, vesicles bud off from its limiting membrane (LM) into its lumen at positions where cargo proteins are concentrated. The incorporation into these intraluminal vesicles (ILVs) separates transmembrane proteins from the cytosol (reviewed by Dobrowolski and De Robertis, 2012). This step is especially important for activated signalling receptors, since they remain active after their endocytosis in the EE. Examples are RTK-receptors, which are inactivated through incorporation into ILVs. The formation of the ILVs is executed by the ESCRT machinery (Henne et al., 2011).

Small GTPases of the Rab family control the fusion of membranes and trafficking of cargo (reviewed by Huotari and Helenius, 2011). These Rabs cycle between the active GTP bound and inactive GDP bound form. They are activated by guanosine exchange factors (GEFs), which exchange GDP with GTP. Upon inactivation through GTPase activating proteins (GAP), Rabs translocate from the LM into the cytosol. Rab5 controls homotypic fusion of EVs with themselves or EEs and initiates ILV formation. These processes are started through recruitment of effectors to the LM of the EE. Among the Rab5 effectors are Hrs, a component of the ESCRT-0 complex, which initiates ILV formation and EEA1, which is required for membrane fusion. Recruitment of both effectors occurs through prior recruitment of Vps34, a PI (3) Kinase, which generates PI(3)P on the cytosolic leaflet of the endosomal membrane. PI(3)P is the docking site that is recognised by the FYVE domains of Hrs and EEA1.

During late stages of endosomal maturation, Rab5 is replaced by Rab7 in a process referred to as Rab conversion (Rink et al., 2005). Rab7 organises fusion of MEs with the lysosome where its cargo is degraded. Initial tethering of both organelles is mediated by the HOPS tethering complex (reviewed by Nickerson et al., 2009). The fusion terminates the life of the ME. Thus, one critical parameter for the lifetime of the endosome is its fusion rate with the lysosome. A delay in fusion increases the lifetime and allows more homotypic fusion between MEs to occur. Hence, it results in an increase in size and numbers of MEs.

Recent work indicates that Mon1 and its C. elegans ortholog Sand-1 are required for Rab conversion in yeast, C. elegans and probably also in mammalian cells (Kinchen and Ravichandran, 2010; Poteryaev et al., 2007; Poteryaev et al., 2010). Loss of function of mon1/sand-1 results in a failure of Rab7 recruitment to the ME. Instead, Rab5 and its GEF Rabex5 accumulate on the LM of MEs. The MEs appear to be dramatically enlarged if monitored with the fluorescent microscope (Poteryaev et al., 2010; Poteryaev et al., 2009). Loss of function of Dmon1 in Drosophila results in an enlargement of maturing endosomes and loss of their association with Rab7. This was the case even when receptor uptake into intraluminal vesicles was suppressed.
2007; Poteryaev et al., 2010). However, an EM analysis that would confirm an enlargement is missing.

While work so far establishes an important role for SAND-1/Mon1 during Rab conversion, its precise function is not resolved. Using C. elegans and mammalian cell culture, Poteryaev et al. (Poteryaev et al., 2010) suggested that Mon1/Sand1 interrupts a positive feedback loop between Rab5 and its exchange factor Rabex5 and then recruits Rab7 to the LM of the MEs. Recent work has established that Rab conversion is also important during phagocytosis in C. elegans (Kinchen and Ravichandran, 2010). In this process, Sand1 appears to bind directly to Rab5GTP. Furthermore, Sand-1 binds to Rab7 only in complex with its binding partner Ccz1. A similar complex formation has been previously observed in yeast (Wang et al., 2002). It was proposed that Rab5GTP recruits Sand1/Mon1, which in turn recruits Rab7 to the EE. It is not clear whether the discrepancy in the suggested mechanisms of Mon1 action is due to the analysis of different processes or due to differences in the experimental approaches. Using the yeast system, the group of Ungermann found that the Mon1–Ccz1 complex acts as a GEF of Rab7 and interacts with the HOPS subunit Vps39 (Nordmann et al., 2010). The interaction of Mon1 with Ccz1 and also Vps39 is mediated through its longin domain. According to their model, Rab5 recruits the Mon1–Ccz1 complex to the EE. The complex then inactivates Rab5 and together with Vps39, recruits and activates Rab7.

Here, we present the analysis of the function of the ortholog of mon1/sand1 in Drosophila, Dmon1. We found that loss of function of Dmon1 results in an enlargement of maturing endosomes and loss of association with Rab7. These endosomes contain Notch and other transmembrane proteins as cargo. Moreover, they appear to mature normally in all aspects tested, despite the loss of association with Rab7. We present evidence that suggest that Dmon1 act in concert with Dccz1. Moreover, we report the first EM analysis of Dmon1- and Dccz1-mutant cells in a multi-cellular organism. We further demonstrate that loss of Rab7 function results in a similar phenotype like Dmon1 and Dccz1 mutants. This suggests that the phenotype of Dmon1 mutant cells can be explained by the loss of association of Rab7 with the LM. Furthermore, we show that recruitment of Rab7 to the endosome is not dependent on the activity of vps34 and therefore probably not on the generation of PI(3)P in the endosomal membrane.

**Results**

**Loss of mut4 and CS084 function results in the accumulation of cargo in enlarged endosomes**

We isolated a mutation we named mut4 as a secondary mutation on a chromosome that bears a bib allele (see also note added at the end and supplementary material). After separation from the bib allele we found that homoyzogosity of mut4 causes the formation of large Notch-positive vesicles (supplementary material Fig. S1). We isolated a second allele, CS084, in a genetic screen. Both alleles are semi-lethal in homzygosity. CS084 caused a weaker phenotype than mut4, indicated by a higher number of escapers in homzygosity.

Using antibodies directed against the extracellular (NECD) and intracellular (NICD) domain of Notch, we found that the vesicles of mut4 cells contained both domains, suggesting that they contain the full-length receptor (Fig. 1A–E). They also contained the transmembrane proteins Patched (Ptc), Ser, Di (not shown), Smootherned (Smo) and the secrected factor Wingless (Wg) (Fig. 1F–J). aPKC, a peripheral protein of the plasma membrane, was not associated with the vesicles (Fig. 1H).

Wg expression is restricted to a small stripe of cells at the dorso–ventral (D/V) boundary of the wing primordium (Fig. 1I, arrow). We detected large Wg-positive vesicles in mutant cells that are located several cell diameters away from its source. Wg is produced in cells at the D/V boundary (highlighted by the arrow in J). Wg can be found in large vesicles of mut4 cells that are located several cell diameters away from its source (J, arrowheads). The Uptake experiment using an antibody directed against NECD together with dextran-Texas-Red revealed that mut4 cells contain enlarged vesicles that are positive for both markers (arrowheads). The region framed in K is shown at higher magnification in L–N.

**Fig. 1. Analysis of the vesicles of mut4 cells.** (A–N) mut4 cell clones were induced either by classical clonal analysis (A–D, same disc) or MARCM (E–N). (A–E) The vesicles of mut4 cells contain the extracellular (NECD) and intracellular (NICD) domain of Notch. Arrows and arrowheads in A–E highlight the clone region. The vesicles contain Ptc (F), Ser (G) and Smo (H), but not the membrane-associated aPKC (H). (I–J) Vesicles are positive for Wg, which is produced in cells at the D/V boundary (highlighted by the arrow in I). Wg can be found in large vesicles of mut4 cells that are located several cell diameters away from its source (J, arrowheads).

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Loss of mut4/CS084 function results in a failure of recruitment of Rab7 to the maturing endosome

For further characterisation, we used several markers for stages of endosomal maturation (Fig. 2). We identified the enlarged endosomes in mut4 cells by co-staining with an anti-Notch antibody. Notch-positive endosomes in wild-type cells contain Rab5 as well as Rab7, indicating that they are MEs (Marois et al., 2006). We found that 43% (n=3 clones; see later) of the Notch-positive mut4 endosomes were positive for Rab5, while this is the case in only 12% of wild-type endosomes (n=3 clones) (Fig. 2A–C, arrows in Fig. 2B). Thus, loss of mut4 function results in an increase of association of Rab5 with endosomes. Moreover, the mut4 endosomes have lost association with Rab7 (0% association; n=3 clones; Fig. 2A,D,E; see later). Consequently, Rab7 was located in the cytosol of mut4 cells (Fig. 2D,E).

These observations indicate that loss of the gene function affected in mut4/CS084 causes a failure of recruitment of Rab7 from the cytosol onto the endosome.

We found that Dor, a core component of the related CORVET and HOPS tethering complexes of Drosophila (Sevrioukov et al., 1999), accumulates on many enlarged endosomes of mutant cells (arrowheads in Fig. 2F). The mutant endosomes were also decorated with Hrs and Spinster, which are markers for MEs (arrowheads in Fig. 2G,H). Like MEs in wild-type cells, the mutant counterparts were labelled by the PI(3)P sensor FYVE-GFP (Fig. 2I). Thus, Rab5 mediated recruitment of Vps34 to endosomes occurs in mutant cells, indicating that Rab5 is active. The majority of the enlarged endosomes were also positive for LAMP1-GFP, a marker for MEs and lysosomes (Fig. 2J,K). These observations indicate that loss of the gene function results in an increase of association of Rab5 with endosomes. Moreover, the mut4 endosomes have lost association with Rab7 (0% association; n=3 clones; Fig. 2A,D,E; see later). Consequently, Rab7 was located in the cytosol of mut4 cells (Fig. 2D,E).

Next, we sequenced the transcribed unit of mut4 and CS084 homozygous flies (Fig. 3A–C). In both of our mutants we found base changes in Dmon1. mut4 is a nonsense mutation which truncates Dmon1 after 70 aa. The truncated Dmon1 is probably not functional, since it lacks most of the protein which truncates Dmon1 after 70 aa. The truncated Dmon1 is probably not functional, since it lacks most of the protein including the longin domain (Fig. 3B,C). This notion is supported by our genetic analysis, which revealed that the phenotype of mut4 in homozygosity is similar to that in transheterozygosity with all three deficiencies (data not shown). The weaker CS084 allele bears a nonsense mutation at position K389 (Fig. 3B,C). The truncated protein includes the longin domain and might therefore have residual activity (Fig. 3C).

Altogether, this analysis indicates that the observed enlarged Notch containing vesicles of mut4 cells are MEs. It also reveals that these MEs appear to mature relatively normally in many aspects, although they have lost the ability to recruit Rab7.

mut4 and CS084 are alleles of the mon1 ortholog of Drosophila

Genetic mapping revealed that the locus is uncovered by the deficiencies Df(2L)Exel6010, Df(2L)Exel8012 and Df(2L)Exel9062. These deficiencies uncover the Drosophila ortholog of yeast Mon1 (here referred to as Dmon1) encoded by CG11926 (Fig. 3A). Dmon1 encodes a 528 aa long protein that possesses a longin domain, which is important for the interactions with its partner Cz1p and also Vps39p in yeast (Nordmann et al., 2010) (Fig. 3C). Dmon1 was an obvious candidate, since loss of its function in C. elegans causes a similar phenotype (Poteryaev et al., 2007). In order to test whether mut4 is an allele of Dmon1, we conducted the following experiments: first, we tested whether loss of Dmon1 produces a similar phenotype like mut4. We depleted cells in the middle region of the wing imaginal discs of Dmon1 function by expressing an RNAi construct with ptcGal4. To achieve maximal depletion, we co-expressed UAS Der2, which enhances RNAi in Drosophila (Dietzl et al., 2007). We found that depleted cells contained enlarged Notch-positive endosomes, which were associated with Rab5 (Fig. 3D,E, arrowhead). Moreover, Rab7 has lost its association with endosomes (Fig. 3F). Thus, the loss of function of Dmon1 produces a phenotype similar to mut4.

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To unambiguously show that mut4 and CS084 are alleles of Dmon1, we generated an untagged UAS Dmon1 construct and performed rescue experiments. When expressed with hhGal4 in the posterior compartment of mut4 wing imaginal disc, UAS Dmon1 causes a normalisation of the size of the Notch-positive endosomes in mutant cells (not shown). For further characterisation, we generated a HA-tagged construct (UAS Dmon1-HA). Like the untagged version, Dmon1-HA was able to rescue the enlarged endosome phenotype of mut4 cells (Fig. 3G,H). Moreover, its expression resulted in the relocalisation of Rab7 to endosomal membranes (Fig. 3G,I,J). The distribution of Rab5 was not affected (Fig. 3K).

Ubiquitous expression of Dmon1 with the daGal4 resulted in a rescue of the lethality of mut4 in homozygosity, giving rise to morphologically normal looking adult flies (not shown). Altogether, these results show that the loss of Dmon1 function is responsible for the phenotype observed in mut4 and CS084 mutants. Thus, the two mutations are alleles of Dmon1 and we renamed them Dmon1mut4 and Dmon1CS084. Since Dmon1mut4 is the stronger (null) allele, we used it for further analysis.

Anti HA staining revealed that Dmon1-HA is distributed throughout the cytosol with no obvious association with endosomes (Fig. 3L,M). The cytosolic localisation is in agreement to what has been reported for sand-1 in most cells in C. elegans (Poteryaev et al., 2007).

Further characterisation of the endosomes of Dmon1 cells

To extend the analysis of Dmon1, we monitored the distribution of key players of the two major recycling pathways in Dmon1mut4 cells. The impact of loss of Dmon1 function on these pathways has not been investigated. Rab4 is a central organiser of the fast and Rab11 for the slow recycling pathway (reviewed by Huotari and Helenius, 2011). In normal cells Rab4 and Rab5 overlap extensively on MEs (Fig. 4A–G, arrows in G). We found that Rab4 is present on most enlarged Dmon1mut4 endosomes (Fig. 4A–G). We quantified the co-localisation of Rab4 and Rab5 with Notch on wild-type and Dmon1mut4 endosomes. We therefore compared the region of a mutant cell clone with an identical wild-type region close by (see Fig. 4H–J). The analysis revealed that Rab4 and Rab5 overlap extensively on MEs (Fig. 4A–G). We quantified the co-localisation of Rab4 and Rab5 with Notch on wild-type and Dmon1mut4 endosomes. 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To assay whether accumulation of Rabex5 occurs on the endosome of Dmon1mut4 cells as it has been observed in C. elegans (Poteryaev et al., 2010), we generated an EOS tagged Rabex5 construct driven by the tubulin promoter (tub. EOS-Rabex5). EOS-Rabex5 was located in the apical region of the cells, overlapping with Rab4 on EEVs (Fig. 4M–P). In contrast to our expectations, the distribution of Rabex5 did not change in the absence of Dmon1 (Fig. 4Q–S).

Rab7 appears to play an essential role also during maturation of autophagosomes (Jäger et al., 2004) and mon1p is required for autophagy in yeast (Wang et al., 2002). We therefore wondered whether Dmon1 has a function during this process. ATG8 is a specific marker of the autophagosomal membrane in all stages of maturation (Shpilka et al., 2011). We used ATG8-GFP to reveal autophagosomes in mut4 mutant cells, but found no effect on distribution and expression of Atg8-GFP in mutant compared to wild-type cells. Moreover, the enlarged Notch-positive endosomes were free of ATG8-GFP (supplementary material Fig. S2K–M).

Overexpression of UAS Dmon1 had no obvious effects on endosomes, neither on their morphology nor their association with Rab5, Rab7, Rabex5 or distribution of Notch (supplementary material Fig. S2A–F). This holds true even if two copies of UAS Dmon1 are expressed (supplementary material Fig. S2G–J).

The phenotype of loss of Dccz1 resembles that of Dmon1

In yeast, Mon1p functions in a complex with Ccz1p (Wang et al., 2002). An orthologous complex appears to act during phagosome maturation in C. elegans (Kinchen and Ravichandran, 2010). A function of Ccz1 during maturation of endosomes in metazoa has not been reported. We therefore analysed the consequences of loss of function of the Drosophila ortholog of ccz1 (here referred to as Dccz1), encoded by CG14980. Using RNAi, we found that the phenotype is very similar to what we observed for Dmon1 (Fig. 5A–F). Loss of Dccz1 resulted in the formation of dramatically enlarged endosomes, which are positive for Notch, Rab4 (Fig. 5A,B,E,F), and Rab5 (data not shown). Localisation of Rab7 on maturing endosomes was dramatically reduced (Fig. 5C,D,F). Like in Dmon1mut4 cells, we observe no change in the subcellular distribution of Rabex5 (Fig. 5G,H).

To determine the subcellular location of Dccz1, we generated an UAS Dccz1-EOS construct. Dccz1-EOS was distributed throughout the cytosol as we have previously observed for Dmon1-HA (Fig. 5I–K). Overexpression of Dccz1 with the Gal4 system did not cause a detectable phenotype (not shown).

The Mon1–Ccz1 complex has been shown to interact with the GTP bound form of Rab5 (Rab5GTP) during phagosome maturation (Kinchen and Ravichandran, 2010). This interaction appears to be mediated by Mon1 (Kinchen and Ravichandran, 2010). We here fail to detect an association of Dmon1/Dccz1 with endosomes. One explanation is that Dmon1/Dccz1 cycles between the cytosol and the endosomal membrane. If the time of association with the endosome is short, only a small fraction of the protein is located at the endosome. This fraction might be too small to be detected. Therefore, we monitored the subcellular distribution of Dmon1 in cells depleted of Dccz1. We reasoned that if its interaction partner is missing, Dmon1 might bind to Rab5GTP at the endosome and becomes trapped there because it cannot form the necessary complex to complete its function. As a result it should accumulate on the endosome. However, we did
cells in more detail, we conducted a transmission electron 

microscope (TEM) analysis (Fig. 6). We first expressed UAS 

Dmon1-RNAi with hhGal4 and UAS Dcr2 in the posterior 

compartment of the wing imaginal disc (Fig. 6A–D). This 

experimental design allowed us to compare (anterior) wild-type 

and (posterior) mutant cells within one disc. We did not observe 

any gross changes in epithelial morphology in mutant territories 

in semi-sections (Fig. 6B). However, we found an enlargement of 

MEs/MVBs in depleted posterior cells (Fig. 6C,D,G,H). We 

observed the same phenotype in Dmon1mut4 cells (Fig. 6E). 

This indicates that the RNAi construct is specific for Dmon1. 

The presence of many ILVs in mutant MVBs indicates that 

the activity of the ESCRT machinery is not affected, as suggested 

by the analysis with the fluorescence microscope. Dmon1 mutant 

cells contained vesicles with the typical morphology of 

lysosomes (arrowhead in Fig. 6E), reinforcing our conclusion 

that lysosome formation is not affected. The mutant cells 

contained more MVBs than wild-type cells: In an area of the 

same size, we found 30 MVBs in Dmon1mut4 discs compared to 

18 in wild-type discs (see supplementary material Fig. S3). This 

finding suggests that the turnover of MVBs, which occurs 

through fusion with the lysosome, is reduced or abolished. MEs 

travel from the periphery of the cells to more perinuclear regions, 

where they fuse with the lysosome. The enlarged MVBs of 

Dmon1 cells were located in perinuclear regions (supplementary 

material Fig. S3). This suggests that their mobility is not severely 

affected by the loss of Rab7 association.

In order to analyse the phenotype of loss of Dccz1 function, we 

expressed UAS Dccz1-RNAi in the posterior compartment of the 

wing imaginal disc. We found similarly enlarged MVBs in the 

depleted posterior cells (Fig. 6F–H). This finding supports the 

notion that Dccz1 acts in concert with Dmon1 in Drosophila. 

We did not find obvious accumulation of autophagosomes in 

Dccz1 or Dmon1 mutant cells.

The phenotype of rab7 cells resembles that of Dmon1 and 

Dccz1 cells

Since we observed a failure of Rab7 to be recruited to maturing 

endosomes in Dmon1 and Dccz1 cells, we were curious whether 

loss of rab7 causes a phenotype that is similar to loss of Dmon1 

function. Therefore, we depleted cells of its function. The 

depletion of Rab7 is very efficient and reduces its expression to 

an undetectable level (Fig. 7A,B). The depletion caused a 

phenotype similar to that of Dmon1. We observed the 

formation of enlarged Notch- and Rab4-positive endosomes 

(Fig. 7A,C–F). Furthermore, the endosomes of depleted cells 

were acidified (Fig. 7G,H). The subcellular distribution of 

Dmon1 was not affected (not shown). Interestingly, we 

observed that many of the enlarged endosomes have lost the 

association with Rab5 (Fig. 7D, arrowheads; cf. Fig. 2).

The EM analysis revealed that rab7 depleted cells contained 

enlarged MVBs as observed for Dmon1 and Dccz1 cells 

(compare Fig. 7I–K with Fig. 6). The results indicate that the 

phenotype caused by depletion of rab7 function resembles that of 

Dmon1 and Dccz1 mutant cells. Thus, a major function of 

Dmon1–Dccz1 is to recruit Rab7 to maturing endosomes.

Dmon1 and Dccz1 act in concert

To find further evidence for a concerted action of Dccz1 and 

Dmon1, we co-overexpressed both together with the Gal4 

system. In contrast to expression of each one alone, we 

observed more and slightly enlarged Notch-positive endosomes

not see any change in the distribution of Dmon1 in Dccz1 

deprecated cells (Fig. 5LM). Moreover, Notch-positive endosomes 

were still enlarged indicating that overexpression of Dmon1 

cannot compensate for the loss of Dccz1 function (Fig. 5LM). 

The same is true for the converse situation (Fig. 5N–P). We next 

looked at the subcellular localisation of Dmon1 in situations 

where the endosomal trafficking is disturbed. Rab5 

overexpression results in the formation of enlarged Notch- 

positive EE (Stenmark et al., 1994). Nevertheless, we did not 

observe any change in the subcellular distribution of Dmon1 

(data not shown).

**EM analysis of Dmon1 and Dccz1 mutant cells**

To characterise the enlarged endosomes of Dmon1 and Dccz1 
cells in more detail, we conducted a transmission electron 

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(Rab7 localisation to the endosome does not require PI(3)P in Drosophila)

Results obtained in C. elegans suggest that Mon1 might be recruited to maturing endosomes through binding to PI(3)P, which is generated by the Rab5 effector and PI(3) kinase Vps34. Thus, a failure in the generation of PI(3)P should result in a failure of Rab7 recruitment, due to a failure of Dmon1 recruitment. We tested recruitment of Rab7 in situations where the PI(3)P generation or access to it by effectors was blocked (supplementary material Fig. S4). The prediction was that if Dmon1 was dependent on PI(3)P, Rab7 recruitment to MEs should be prevented in these situations. First, we expressed an RNAi construct of vps34 and found that Rab7 is still associated with endosomes (data not shown). Secondly, we expressed up to three copies of GFP-FYVEx2 (supplementary material Fig. S4A–E). It consists of two PI(3)P binding FYVE-domains fused to GFP (Wucherpfeppen et al., 2003). UAS GFP-FYVEx2 is associated with endosomes if expressed with the Gal4 system (supplementary material Fig. S4A–E). We surmised that strong expression of this construct saturates most PI(3)P binding sites on the maturing endosome and therefore should prevent binding of Dmon1. In accordance with this notion, expression of three copies of this construct (3×GFP-FYVEx2) in wing disc cells caused the formation of enlarged Notch-positive endosomes (supplementary material Fig. S4D,E). Nevertheless, we found that many are associated with Rab7 (supplementary material Fig. S4D,E). Thirdly, we suppressed the activity of Vps34 by expressing a kinase dead version, which acts in a dominant negative manner (Vps34KD). Expression of this construct results in a strong loss of GFP-FYVEx2 from endosomes (Juhaš et al., 2008), supplementary material Fig. S4F,G). In addition we found that the cells contain enlarged MEs with a strongly reduced number of

(Depletion of Dccz1 in Dmon1mut4wing imaginal discs with hhGal4 had no effect on the morphology of Notch-positive endosomes (data not shown). This finding further supports the notion that both proteins act in concert.)
This phenotype was expected since Hrs, which initiates ILV formation, is recruited to the endosome through binding to PI(3)P through its FYVE-domain (Hurley, 2010). Altogether, these observations indicate very efficient inhibition of PI(3)P formation by Vps34 KD.

Expression of this construct resulted in the formation of enlarged Notch-positive endosomes, which are located apically. However, they were still associated with Rab7 (supplementary material Fig. S4H–K). Last, we monitored the distribution of Rab7 in cells mutant for the null allele vps34Dm22 (supplementary material Fig. S4M–O). Again, we observed enlarged Rab7-positive endosomes in mutant cells (supplementary material Fig. S4M–O, arrows in N,O). These results suggest that formation of PI(3)P is not necessary for the Dmon1 mediated recruitment of Rab7 onto the endosome in Drosophila. Interestingly, the loss of function of vps34 does not result in the ectopic activation of Notch, although the formation of ILVs is suppressed (not shown).

Accumulation of signalling receptors combined with a failure of ILV formation does not result in activation of signalling pathways

Given the importance of the endosomal pathway in regulation of signalling pathways, it was surprising for us that the few Dmon1mut4 homozygous flies that escaped to the adult stage did not display any obvious patterning defects. This suggests that cell signalling during pattern formation is not affected. This conclusion was confirmed when we monitored the expression of target genes of several signalling pathways in Dmon1 wing discs (supplementary material Fig. S5). We monitored the activity of the EGF-, Wg-, Dpp-, and Notch pathways using the expression of argos-lacZ, Dfz3-lacZ, omb-lacZ and Gbe+Su(H)-GFP, respectively. None of these markers were affected in their expression. Moreover, we did not observed any change of the markers if ILV formation was suppressed in Dmon1 cells by concomitant removal of hrs function (supplementary material Fig. S5). These results indicate that incorporation into ILVs is not the only mode of inactivation of active signalling receptors in these cells.

Discussion

Here, we report the characterisation of the Drosophila ortholog of Mon1/Sand-1, Dmon1, and its partner Dccz1. We show that Dmon1 is required for the recruitment of Rab7 to the maturing endosome as has been previously observed for the orthologs in C. elegans and yeast. Our experiments support the notion that Dmon1 and Dccz1 act in concert to fulfil their function in metazoans. This is in agreement with the finding that both proteins act in concert in yeast and during phagosome maturation in C. elegans (Kinchen and Ravichandran, 2010). We here provide evidence that this is true also during endosomal trafficking of cargo in metazoans. We present the first EM analysis of Dmon1 and Dccz1 in a metazoan and show that the loss of function of each gene results in the same phenotype.
formation of more and dramatically enlarged MEs with a high number of ILVs. We extended the characterisation of the endosomes in mon1 mutants using additional markers. We found that the endosomes acidify and the adapter lipid PI(3)P is generated in their limiting membranes, indicating that Rab5 is active and its effectors can be recruited to the endosome. In agreement with this notion, we observe association of Hrs and FYVE-GFP with the enlarged endosomes. These markers are recruited to the LM through binding to PI(3)P. The recruitment of the ESCRT-0 component suggests that ILV formation is initiated. This conclusion was confirmed by our EM analysis where we found that the enlarged MEs of Dmon1 cells are MVBs with many ILVs. In our eyes these results are remarkable, since it indicates that the main aspects of endosome maturation occur in absence of Rab7 function and thus, appear not to be coordinated with the processes mediated by Dmon1 and Rab7.

Based on experiments in mammalian cell culture, it has been proposed that RILP, an effector of Rab7, is involved in ILV formation and interacts with ESCRT-II besides its established function in mediating the movement of the ME to perinuclear regions (Progida et al., 2007). Our results indicate that ILV formation is not significantly affected in Dmon1 or Rab7 mutant cells. Thus, the function of RILP during ILV formation is either independent of Rab7, or RILP has no such function in an integral tissue or in cells in Drosophila. Furthermore, RILP has been shown to be required for endosomal motility in mammalian cell culture cells (Cantalupo et al., 2001). We observed that, like in the wild type, the MEs of Dmon1 cells move to a perinuclear region. Thus, they can move at least to a certain degree in the absence of RILP recruitment by Rab7. Either endosomes can move in an alternative manner in cells or a functional tissue or the mobility is regulated differently in Drosophila. In contrast, the enlarged endosomes in cells depleted of vps34 function were located close to the apical cell membrane, indicating that their transport to the perinuclear region is severely affected. Thus, it appears that the function of Vps34 is required for the movement.

Our TEM analysis shows that depletion of Rab7 results in a similar defect like loss of Dmon1 function. Hence, the failure to localise Rab7 to the endosome explains the phenotypes of Dmon1 and Dccz1 cells: Rab7 is required for fusion of MEs with the lysosome (Huotari and Helenius, 2011). Consequently, loss of Rab7 function leads to a loss of ME turnover and, hence, to an increase of the number of MVBs in a cell. Moreover, the extended lifetime of MEs allows more Rab5 mediated homotypic fusions to occur, which result in the enlargement of MEs. We observe all these phenotypes in our EM analysis of rab7, Dmon1 and Dccz1 cells. The similarity
of the phenotypes of these cells also indicates that the recruitment of Rab7 to the endosomal membrane is pivotal for its function.

While many of our findings in agreement with previous reports, there are also differences. We did not find evidence for a role of PI(3)P for the recruitment of Dmon1 to the endosome, as has been proposed for C. elegans (Poteryaev et al., 2010). We observed that the loss of function of Vps34 and therefore loss of PI(3)P does not prevent recruitment of Rab7 to MEs. Thus, the formation of PI(3)P is probably not involved in its recruitment. This is in agreement with previous work in mammalian cell culture where the role of PI(3)P in Rab7 recruitment to maturing phagosomes was investigated. In this case treatment with PI(3)P Kinase inhibitors failed to prevent Rab7 recruitment onto phagosomes (Vieira et al., 2003).

We observed that Rab5 does not accumulate on endosomes in the absence of Dmon1 or Dccz1. Rather, we find that Rab5 is eventually lost from a fraction of the enlarged endosomes. Nevertheless, the fact that we observed enlarged endosomes in cells mutant for Dmon1 or Dccz1, suggests that more fusion events between endosomes occur. Assuming that these fusions are mediated by Rab5, a potential explanation for the enlarged endosomes is that the transient association of Rab5 with the endosomal membrane is prolonged in mutant cells. This is supported by our finding that the fraction of Rab5-positive endosomes is increased in Dmon1mut4 cells. Thus, a crucial common parameter in C. elegans and Drosophila is the longer perdurance of Rab5 on the ME, which occurs in both species. The accumulation of Rab5/Rabex5 might be a species-specific effect.

We found that co-overexpression of Dmon1 and Dccz1 results in an increase of Rab5 associated endosomes. Thus, in contrast to C. elegans, the association of Rab5 with endosomes is maintained rather than weakened, as would be expected if the complex were a negative regulator of the Rab5/Rabex5 loop. Thus, this finding does not support a role of the Dmon1–Dccz1 complex in inactivation of the Rabex5/Rab5 loop.

The observed differences in Drosophila versus C. elegans can be explained by the analysis of different species and/or analysis of different tissues. However, this explanation would imply different mechanisms of Dmon1–Dccz1 action in different species. Another possibility is that the differences in the results reflect the analysis of different types of tissues. We analysed the function in an epithelium whereas the analysis in C. elegans were largely done in oocytes and coelomocytes. This notion is supported by the differences in results obtained in C. elegans if oocytes and coelomocytes on the one hand and phagocytes on the other are analysed (see Introduction). More work is required to clarify these issues.

To our surprise, autophagy appears not to be affected by loss of Dmon1, in marked contrast to the yeast Mon1p mutant phenotype (Wang et al., 2002). Thus, if Rab7 is involved in autophagosomal maturation in Drosophila, it must be recruited to the autophagosome in a Dmon1 independent manner. It is possible that the few weakly Rab7-positive vesicles we occasional observe in Dmon1 cells are autophagosomes. The function of yeast Mon1p appears to differ in certain aspects from Mon1 of metazoans, since Sand1 can only partially rescue the yeast Mon1p mutant (Poteryaev et al., 2007). In addition yeast Mon1p is not able to rescue Dmon1 mutants of Drosophila (J.Y. and T.K., unpublished results). In this light it is not surprising to find differences in the function of Dmon1 compared to Mon1p.

### Activation of signalling pathways during delayed endosomal trafficking

Loss of function of ESCRT complexes I to III results in over-activation of several signalling pathways, among them the Notch, RTK and Dpp pathways (Huotari and Helenius, 2011). A major function of these complexes is to incorporate the activated signalling receptors into ILVs of MEs. This function is thought to attenuate signalling since the intracellular domain of activated receptors becomes sequestered from the cytosol (Dobrowolski and De Robertis, 2012). Thus, one explanation of how loss of ESCRT function leads to unregulated signalling is that the activated receptors are not sequestered from the cytosol through the failure of ILV formation. In the case of RTK and Dpp signalling this could lead to prolonged signalling. In the case of Notch, perdurance of the receptor in the limiting membrane could lead to activation through alternative ecto-domain shedding mediated by the activated proteases in the ME. We tested this explanation by monitoring situations where ILV formation and endosome turnover is suppressed. We failed to observe ectopic activation or over-activation of signalling pathways in Dmon1 cells, even when ILV formation was disturbed. Thus, the activated receptors still become sequestered from the cytosol despite their accumulation. We were surprised that the concomitant loss of Dmon1 and hrs function fails to affect cell signalling. While loss of hrs function does not result in Notch activation, it has been shown to activate RTK signalling in several tissues (Jékely et al., 2005; Jékely and Rorth, 2003; Lloyd et al., 2002). We expected this effect to be enhanced in cells where the lifetime of the endosomes is dramatically prolonged and receptors accumulate to higher levels than normal. However, we do not observe ectopic or stronger activation of the RTK signalling, neither upon hrs, nor Dmon1 hrs loss of function. In addition, we did not observe an effect on the activity of the other pathways tested, such as the Dpp and Notch pathway, which have been shown to become activated upon inactivation of ESCRT-I–III complexes (Moberg et al., 2005; Thompson et al., 2005; Vaccari and Bilder, 2005). Likewise, we did not observe ectopic activation upon loss of vps34 function. In this case we observe a strong reduction of formation of ILVs, similar to loss of hrs function (Lloyd et al., 2002). Altogether, the results suggest that the failure of incorporation of activated receptors in ILVs is not sufficient to explain the uncontrolled signalling of various pathways observed in ESCRT mutants. Additional mechanisms must be involved.

### Note

It should be noted that the Dmon1mut4 allele is a background mutation present on some bib+ chromosomes, and it accounts for the aberrant protein trafficking phenotype that was erroneously attributed to bib+ in a previous study (Kanwar and Fortini, 2008). The attribution of this phenotype to bib+ was initially made based on rescue of the phenotype by a 16.5 kb bib+ wild-type genomic transgene. However, subsequent retesting of this bib+ rescue fragment established that it does not rescue the protein trafficking phenotype (R. Kanwar and M.F., unpublished data), which instead is caused by the unlinked Dmon1mut4 mutation as reported in the current study.

### Materials and Methods

**Drosophila genetics**

UAS lines used were: UAS Dmon1RNAi (VDRC 38600, 103378), UAS Ccz1RNAi (VDRC 19479), UAS Vps34RNAi (VDRC 100269). UAS-Mon1, UAS-Mon1HA, tubRab4-mCherry were generated in this work. Gal4 lines used in this
Clonal analysis

Clones were generated with the FLP/FRT system (Xu and Rubin, 1993) and induced in first instar larvae (24–48 hours after egg laying) by applying a 70-minute heat shock (37°C). Dmon1+/- MARCM clones were induced with hsFlp1.22 tubGal4 UAS nlsGFP; PRT40A Gal80/Dmon1+/- FRT40A larvae.

Uptake assay

Uptake assays were described as (Le Borgne et al., 2005) (Texas Red dextran, MW3000, Molecular Probes, D-3328).

Immunostaining and microscopy

Antibody staining was performed according to standard protocols. Discs were stained in addition with the nuclear marker Hoechst 33258, anti-Wg antibody (4D4), the mouse Notch antibodies against the extracellular (C458.2H) and transmembrane regions (21C8), anti-Rab5 (a gift of Marcos Gonzalez-Gaitan); and anti-HA (Roche); anti-Hrs (Lloyd et al., 2002); anti Ser (a gift from Elisabeth Knust). Fluorochrome-conjugated secondary antibodies were purchased from Invitrogen/Molecular Probes. Images were obtained with a Zeiss Axiosimager Z1 Microscope equipped with a Zeiss Apotome.

Constructions

All PCR-amplifications were performed with the Expand HF-PCR Kit (Roche). PCR-products were extracted from agarose gel by E.Z.N.A. Gel Extraction Kit (Omega Bio-Tek Inc., USA). The UAS Dmon1HA construct was generated by PCR using a Dmon1-cDNA (NCBI Ref. Seq. No. NM_133024.1, synthesized by GenScript USA Inc.). Amplified DNA-fragments were cloned into the pUAS-pattB-Vector (Bischof et al., 2007) using the Not-site. UAS Dmon1 was generated by Not cloning into the pattB-UAS vector. UAS Dccz1-EOS (EOS-tag at C-terminus) construct was generated by splice over extension PCR (Horton et al., 1990) using Gold-Clone RH02365 (DGRC) and ECS. ccz1-EOS was cloned into pattB-UAS vector by Not. tub.erb3-EOS (EOS-tag at the N-terminus) construct was generated by splice over extension PCR from Gold-Clone SD03358 (DGRC) and EOS. erb3-EOS was then cloned into pCasp-tubPromoter3-UTR.

tub.rab4-4-mCherry (mCherry-tag at N-terminus) construct was generated by splice over extension PCR from EST-Clone RE40706 (DGRC) and mCherry-cDNA (pAB118-mCherry) and cloned into pCasp-tubPromoter3-UTR.

EM analysis

Wing discs were fixed in 2.5% glutaraldehyde in 100 mM phosphate buffer (pH 7.4) containing 2% sucrose. Following 2x washes in 0.1 M cacodylate buffer, specimens were dehydrated in EtOH and embedded in araldite using acetone as intermediate solvent. Thin sections were stained with 2% uranyl acetate and 0.5% lead citrate. Sections were observed under an EM 902 (Zeiss) microscope at 80 KV.

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Author contributions

I.Y. was involved in all experiments (performed the major works); T.T. provided the control experiments for the Rab7 analysis. F.G. helped with the electron microscopy. M.F. and T.S. provided the Dmon1 allele CS084 T.K. is the supervisor of the project.

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Supplementary material available online at


References


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Fig. S1. The bib<sup>105</sup> chromosome carries a second mutation that causes the formation of increased Notch positive vesicles in imaginal disc cells. (A, B) bib function is not required for Notch signalling along the D/V boundary of the wing. The arrow highlights the region where bib function is lost (labelled by loss of GFP). However, the expression of the Notch target gene wg is not affected in mutant cells. (C-G) Clone of the bib<sup>105</sup> chromosome induced by MARCM. Arrow points to the mutant region, which is labelled through expression of GFP and shown in higher magnification in (E-G). In the clone area Bib is absent (D, F, G, arrows). The cells contain large vesicles that contain Notch (E, G). In contrast, in clones induced with the bib<sup>105</sup> chromosome (arrows in I, J), Bib is still absent in mutant territories (H), but the cells do not contain large Notch positive vesicles (I). Expression of UAS bib in bib<sup>105</sup> clones induced by MARCM, does not prevent formation of the large Notch positive vesicles (K-P). (Q-T) Clones induced with the separated mut4 mutation. The cells contain the large Notch positive vesicles (Q, S, T), but do express Bib normally (R, S). Note, that a fraction of Bib is located in the large vesicles of mut4 cells (T, arrows). (U, V) Wing imaginal disc of a mut4 mutant fly. All cells contain the large Notch positive vesicles.
Fig. S2. Over-expression of UAS Dmon1-HA has no effect on distribution of endosomal key proteins. (A-F) Expression of one copy and (G-J) of two copies of UAS Dmon1-HA. The distribution of Notch (B, H), Rab5 (C, G), Rab7 (D) and Rabex5 (F) is not affected.
Fig. S3. Loss of Dmon1 function results in the accumulation of MVBs in cells. (A, B) Wildtype and mutant areas are shown at the same magnification. The MVBs are highlighted in yellow colour. While Dmon1 cells have 30, wildtype cells have only 18 MVBs in the same area. Thus, Dmon1 cells have more MVBs than their wildtype counterparts indicating that the turnover of endosomes is disturbed.
Fig. S4. The role of PI(3)P and Vps34 in recruitment of Rab7 to the endosome. (A-C) Expression of an UAS FYVE-GFP construct in wildtype discs, using ptcGal4. FYVE-GFP binds to PI(3)P containing MEs (B), which are positive for Notch (B, C). The arrow in (B) points to one of the FYVE-GFP associated MEs. (D, E) Expression of three copies of the UAS FYVE-GFP construct with enGal4 results in the formation of enlarged endosomes in cells of the posterior compartment, which are positive for Rab7 (arrows in E). (F, G) Upon co-expression of the kinase dead version of Vps34 (Vps34\textsuperscript{kd}), FYVE-GFP fails to be recruited to the endosomes. This phenotype indicates that the expression of Vps34\textsuperscript{kd} efficiently prevents the formation of PI(3)P. Nevertheless, cells expressing Vps34\textsuperscript{kd} still contain Rab7 positive endosomes (H-K). These endosomes are concentrated at the apical side of the imaginal disc epithelial cells (arrow in I-K). (L) TEM analysis of Vps34\textsuperscript{kd} expressing cells. The cells contain many enlarged MEs that contain only few ILVs (arrows). (M; O) Clonal analysis of the null allele of vps34, vps34\textsuperscript{dm22}. Mutant cells contain Rab7 positive endosomes (arrows in N, O). Altogether, these results indicate that Rab7 is recruited to the endosome in a Vps34 independent manner.
Fig. S5. Concomitant loss of *hrs* and *Dmon1* function does not impact on signalling of RTK, Dpp, Wnt and Notch pathways. The effect of *Dmon1* loss of function on the expression of the reporter genes is monitored either in *Dmon1* mutants or by clonal analysis.