Localization and mislocalization of the interferon-inducible immunity-related GTPase, Irgm1 (LRG-47) in mouse cells

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Abstract

Irgm1 (LRG47) is an interferon-inducible Golgi membrane associated GTPase of the mouse whose disruption causes susceptibility to many different intracellular infections. Irgm1 has been variously interpreted as a regulator of homologous effector GTPases of the IRG family, a regulator of phagosome maturation and as an initiator of autophagy in interferon-induced cells.

We find that endogenous Irgm1 localises to late endosomal and lysosomal compartments in addition to the Golgi membranes. The targeting motif known to be required for Golgi localisation is surprisingly also required for endolysosomal localisation. However, unlike Golgi localisation, localisation to the endolysosomal system also requires the functional integrity of the nucleotide binding site, and thus probably reflects transient activation. Golgi localisation is lost when Irgm1 is tagged at either N- or C-termini with EGFP, while localisation to the endolysosomal system is enhanced. N-terminally tagged Irgm1 localises predominantly to early endosomes, while C-terminally tagged Irgm1 localises to late endosomes and lysosomes. Both these anomalous distributions are reversed by inactivation of the nucleotide binding site, and the tagged proteins both revert to Golgi membrane localisation.

Irgm1 is the first IRG protein to be found associated with the endolysosomal membrane system in addition to either Golgi (Irgm1 and Irgm2) or ER (Irgm3) membranes, and we interpret the result to be in favour of a regulatory function of IRGM proteins at cellular membrane systems. In future analyses it should be borne in mind that tagging of Irgm1 leads to loss of Golgi localisation and enhanced localisation on endolysosomal membranes, probably as a result of constitutive activation.

Introduction

The interferon-inducible immunity-related GTPases (IRG proteins, previously named p47 GTPases) are resistance factors against intracellular pathogens in mice. Irgm1 has been reported to be a uniquely powerful

member of the IRG family, because mice deficient in this gene showed complete loss of resistance to all bacterial and protozoal pathogens tested so far (reviewed in [Martens, 2006 #3142] [Taylor, 2007 #3521]). Several other IRG proteins have also been shown to be required for complete resistance to *Toxoplasma gondii* (Irgm3, Irgd, Irgb6) (reviewed in [Zhao, 2009 #3607]) or *Chlamydia trachomatis* (Irgm3 and Irgb10) [Bernstein-Hanley, 2006 #3165;Coers, 2008 #3535], but resistance functions against other pathogens have not been documented for these proteins. The resistance mechanisms of IRG proteins are still unclear. In the case of resistance to *T. gondii*, which has been most comprehensively documented, interest is focused on the unexplained ability of IRG proteins to cause disruption of the parasitophorous vacuole, resulting in the death of the parasite in the cytosol [Martens, 2005 #3082] [Zhao, 2009 #3509]. Since, alone of the IRG proteins assayed, Irgm1 has never been found at the parasitophorous vacuole, it is clear that this IRG protein is not directly involved in the observed vesiculation and disruption of the parasitophorous vacuole membrane. It has, however, been shown that Irgm1, with its closest homologues Irgm2 and Irgm3, is required for regulating the GTPase cycle of the other IRG proteins in the cytoplasm [Hunn, 2008 #3269]. The general susceptibility of Irgm1-deficient mice to bacterial and protozoal infection can be accounted for as a regulatory failure that disables the IRG-dependent resistance mechanism with cytopathic consequences. Irgm1-deficient mice show IFNg-dependent haemopoietic (Goodell) and lymphoproliferative (sher) defects and there is now direct evidence that the unregulated expression of the remaining IRG proteins is responsible for the cellular injury (Taylor). Other mechanisms have, however, been proposed for the role of Irgm1 in disease resistance, especially in the case of resistance to *Mycobacteria*, which is lost in Irgm1 deficiency. Proposed mechanisms for Irgm1 in cell-autonomous resistance include: accelerated lysosome fusion and/or acidification of phagosomes [MacMicking, 2003 #3010] [Deghmane, 2007 #3610], and induction of autophagy [Gutierrez, 2004 #3156] [Singh, 2006 #3144].

There are about 18 genes encoding interferon-inducible IRG proteins in the C57BL/6 genome. The proteins have been divided into two major sequence subfamilies, informally designated GKS and GMS [Bekpen, 2005 #3131]. GMS subfamily members Irgm1, Irgm2 and Irgm3 have a most unusual methionine (GX4GMS) in the position of an otherwise universally conserved lysine (GX4GKS) in the P-loop of the G1 motif of the nucleotide-binding site. Unlike the GKS protein, Irga6, for which a crystal structure is available [Ghosh, 2004 #3034], the GMS subfamily proteins have proved difficult to purify and little is known of their structural or biochemical properties. Nevertheless there is suggestive evidence from immunoprecipitated material and from fusion proteins that, despite the remarkable modification in the G1 motif, the GMS subfamily proteins are able to bind and hydrolyse GTP[Taylor, 1996 #2040].

The expression of Irgm1, together with other IRG proteins (except Irgc), is strongly induced by interferons [Bekpen, 2005 #3131]. In IFNγ-induced cells, the IRG proteins associate with cellular membranes to different extents. Irgm1 is exceptional among IRG proteins assayed in that it is exclusively membrane-associated, with no detectable cytosolic pool. Although no transmembrane domain has been identified within the Irgm1 sequence, the protein behaves like an integral membrane protein in resistance to extraction with high salt, sodium carbonate or urea. By immunofluorescence, native, IFNγ-induced Irgm1 localizes strongly to Golgi membranes and to a lesser extent to unidentified cytoplasmic membrane systems. The Golgi localization depends on a predicted amphipathic helix in the C-terminal domain of the protein, the αK-helix, but the basis for localization to other cytoplasmic membrane systems has not been studied. Upon latex-bead phagocytosis, Irgm1 rapidly re-localizes in both fibroblasts and macrophages to F-actin-rich plasma membrane ruffles associated with phagocytic cups, and remains associated with the phagosomes as they mature into phagolysosomes and become LAMP1 positive [Martens, 2004 #3026].

In this study we examine in detail several aspects of the localization of Irgm1 to cytoplasmic compartments. Building on the earlier observation that the 20-residue amphipathic αK helix of Irgm1 is sufficient to target EGFP to the Golgi apparatus, we show that this sequence can also target EGFP to a cytoplasmic membrane system identified as lysosomes and late endosomes. We show that the αK helix could be also used in the form of a synthetic biotinylated peptide to target a labelled streptavidin complex to Golgi membranes in fixed, permeabilized cells. This biotin-peptide probe also stained cytoplasmic organelles outside the Golgi that we were able to show corresponded to late endosomes and lysosomes. This novel association proved to reflect the previously documented diffuse cytoplasmic localization of native, IFNγ-induced Irgm1, which had not been located to a specific compartment. Irgm1 has a constitutive association with the endolysosomal membrane system in addition to the Golgi apparatus, a unique property among IRG proteins tested so far. The lysosomal localization of Irgm1 is mediated by the same amphipathic helix that mediates Golgi targeting. We observed earlier that N-terminally EGFP-tagged, and C-terminally FLAG-tagged Irgm1 both mis-localize in cells, but the observations were not followed up and the compartments involved were not identified[Martens, 2004 #3026]. We now show that N and C terminal EGFP tags on Irgm1 both lead to loss of Golgi localization and localization to other membrane compartments. The mis-localization of tagged Irgm1 is dependent on nucleotide binding, and we suggest that Golgi localization is a default state probably associated with an inactive GDP-bound state of the protein. The mis-localization of C-terminally tagged Irgm1 suggests that functional experiments employing this construct in studies of lysosome fusion and autophagy [Deghmane, 2007 #3610] [Gutierrez, 2004 #3156] [Singh, 2006 #3144] may now need to be interpreted in terms of the activation state of the protein.

Results

IFNγ-induced Irgm1 localizes to Golgi apparatus and late endocytic/

lysosomal compartments

We previously reported that IFNg-induced Irgm1 (LRG-47) is exclusively membrane-bound and localizes in large part to the Golgi apparatus in L929 fibroblasts, TIB-75 hepatocytes and Raw 264.7 macrophages. This localization is mediated by an amphipathic helix near the C-terminus [Martens, 2004 #3026]. We noted that IFNγ-induced Irgm1 has a further distributed signal throughout the cell periphery of which the subcellular localization remained to be identified. In the present study, we employed the microscopically favorable murine embryonic fibroblasts (MEFs) to characterise the intracellular localisation of Irgm1 in more detail.

In IFNγ treated MEFs, both goat polyclonal anti-Irgm1 antiserum P20 and the mouse monoclonal anti-Irgm1 antibody 1B2 gave a focused adnuclear signal in all cells and additional distributed granular signals, more or less conspicuous in different cells. As previously reported [Martens, 2004 #3026], the adnuclear signal accurately overlapped with both *cis*-Golgi matrix protein GM130 (Figure 1A panels a-c) and *trans*-Golgi/*trans*-Golgi network protein TGN38 (Figure 1A panels d-f). Less accurate co-localization was observed with cation-independent mannose 6 phosphate receptor (CI-M6PR), which is predominantly localized in late endosomes (Figure 1A panels g-i); Irgm1 localizes rather to places adjacent to or partially overlapping with CI-M6PR positive compartments. Furthermore, the more widely distributed signal of Irgm1 accurately co-localized with the late endosome/lysosome marker LAMP1 (Figure 1B panels a-d)). By comparison, Irgm2 localizes exclusively to Golgi apparatus, as reported earlier {Martens, 2006 #3142} with no extra signal overlapping with LAMP1 (Figure 1B panel c). To confirm the apparent lysosomal localization of Irgm1, the acidotropic dye LysoTracker was loaded and cells were co-stained with anti-Irgm1 antibody 1B2. Irgm1 was found to accumulate around the LysoTracker enriched compartments (Figure 1B panel e-g). By comparison, endogenous Irgm1 rarely co-localized with transferrin-labelled early and recycling endocytic compartments (Figure 1C). Taken together, we conclude that native, IFNγ-induced Irgm1 is associated with acidified late endocytic/

lysosomal compartments in addition to Golgi apparatus.

We previously reported that a predicted amphipathic helix near the C terminus of Irgm1 (named αK helix by homology to the equivalent element in the known Irga6 structure) is responsible for the localization of the protein to Golgi membranes [Martens, 2004 #3026]. The αK helix is a true targeting motif since, as a C-terminal tag, it was sufficient to target EGFP accurately to the Golgi apparatus as defined by GM130 staining. Disruption of amphipathicity by glutamate insertion into helical region abolished the Golgi localization in both EGFP-Irgm1 αK construct and Irgm1 full-length protein. Since we now show that IFNγ-induced Irgm1 also localises to lysosomes, we ask whether the native αK helix of full-length Irgm1 is also necessary for lysosomal localization. Full length Irgm1 and the ins 362,367E Irgm1 mutant, in which the amphipathicity of the αK targeting helix was destroyed by two glutamate insertions [Martens, 2004 #3026], were transiently expressed in un-induced MEFs (Figure 2). Wild type Irgm1 showed both Golgi and lysosomal association like the endogenous IFNγ-induced protein (Figure 2 panels a-c). In these cells the co-localization with LAMP1 was even more striking than in IFNγ-induced cells. Irgm1 ins 362, 367E mutant was distributed as dotty structures throughout the cytoplasm that showed neither Golgi nor lysosomal localization (Figure 2 panels d-f and [Martens, 2004 #3026]) and were not further identified. Thus the native amphipathic αK helix is required for both Golgi and lysosomal targeting of full length Irgm1 protein.

The amphipathic αK helix near the C-terminus of Irgm1 shows Golgi and lysosomal targeting property.

We previously observed that the EGFP-Irgm1 αK construct associated with a cytoplasmic compartment in addition to Golgi-marker-positive compartments in some transfected cells (Martens et al, 2004). We therefore now analysed the intracellular localization of the EGFP-Irgm1 αK construct

in more detail in MEFs (Figure 3). In 46% of the transfected cells, the EGFP-Irgm1 αK construct indeed localized primarily to the Golgi apparatus as shown by co-staining of GM130 (Figure 3 panels a-i). However, in other transfected cells (54%), EGFP-Irgm1 αK localized primarily to punctate or vesicular structures distributed throughout the cytoplasm. Many of those structures accurately co-localized with the lysosomal protein LAMP1 (Figure 3 panels j-o). In 14% of transfected cells, EGFP-Irgm1 αK showed both Golgi and lysosomal localization. Thus the αK amphipathic helix can target EGFP *in vivo* to both Golgi and lysosomal compartments.

These experiments confirmed that the mixed Golgi and lysosomal localization shown by EGFP-Irgm1 αK reflects properties of the full-length Irgm1 protein. Since native Irgm1 is rapidly recruited to latex-bead phagosomes [Martens, 2004 #3026], we asked whether the αK helix is also responsible for phagosomal accumulation of Irgm1. EGFP-Irgm1 αK was expressed by transfection in MEFs and cells were incubated with 2-μm latex beads to induce phagocytosis. Even though co-localization was seen between EGFP-Irgm1 αK and LAMP1 in cells taking up beads (Figure 4 panels e, f, g arrows), latex bead phagosomes remained negative for EGFP-Irgm1 αK (Figure 4 panels a, e, i). Thus the Irgm1 αK helix alone is not sufficient for the active accumulation of full-length Irgm1 on the phagosome.

The overall amphipathicity of the αK helix and the identity of the non-hydrophobic residues both contribute to the Golgi and lysosome localization of the αK helix.

To investigate the possible mechanisms of subcellular localization of Irgm1, an alanine mutagenesis scan was performed based on the targeting construct EGFP-Irgm1 αK. Single mutations of hydrophobic residues (C356A；I358A；V359A；F362A；F363A；L365A；L366A not shown) double mutations (F362A F363A and L365A L366A not shown) and even the

quadruple mutant (F362A F363A L365A L366A, Figure 5A) had no influence on EGFP-Irgm1 αK localization. Single mutations of the hydrophilic residues also had no effect on localization (N360A； R364A； R367A Figure 5B). However, if more than one charged or polar residue was mutated to alanine both Golgi and lysosomal localization were completely abolished (N360A, R364A; R364A, R367A; N360A, R364A, R367A; Figure 5C). These latter mutants were distributed in an unspecific reticular structure throughout the cytoplasm. These results suggest that both the overall amphipathicity of the αK helix and the identity of the non-hydrophobic residues contribute together to the membrane and Golgi/lysosome localization of the αK helix, and it is possible that the specificity for Golgi and lysosomal membranes is determined by the identity of the polar residues.

Synthetic αK amphipathic peptide mimics the localization of endogenous Irgm1.

Biochemical studies showed that Irgm1 behaves like an integral membrane protein, even though no transmembrane domain has been identified within the protein sequence. It was also demonstrated that Irgm1 exclusively associates with the membrane fraction of the cell [Martens, 2004 #3026]. The attempts to purify recombinant Irgm1 protein failed due to insolubility (not shown). To investigate the possible mechanisms of Irgm1 localization, a peptide corresponding to the targeting amphipathic sequences (SK*LRLMTCAIVNAFFRLLRFLPCVCC) of Irgm1 was synthesized. The lysine in position 2 (K*) was covalently conjugated with biotin. Thus the peptide could be loaded onto fluorochrome-conjugated streptavidin protein, which has four biotin binding sites, to form a tetrameric, fluorescent peptide-streptavidin complex. The localization of this complex was then examined directly on fixed, permeabilized cells. The detailed procedures for making and using this tetramer system are described in Materials and Methods. Figure 6 shows the staining pattern of Cy3 labelled streptavidin-Irgm1 αK

tetrameric complexes in MEFs. Cy3-labeled streptavidin alone was used as control and pictures were taken with the same exposure time (Figure 6 panels a, b). The streptavidin-Irgm1 αK complex staining shows both perinuclear and vesicular structures corresponding to the localization of endogenous Irgm1 protein (Figure 6 panels c, d, g, h). GM130 (Figure 6 panel e) and LAMP1 (Figure 6 panel i) were additionally used to identify the Golgi apparatus and lysosomes. Streptavidin-Irgm1 αK complexes showed striking co-localization with full length Irgm1 at both Golgi apparatus and lysosomes. There was, however, more diffused staining with the synthetic complexes, perhaps due to the intrinsic hydrophobic character of the peptide. These results demonstrate that the streptavidin-αK peptide complex closely resemble the localization of endogenous Irgm1 and provides a valuable novel method to investigate the function of targeting motifs of proteins.

Both N and C terminal EGFP-tags lead to the mislocalization of Irgm1

The proposed autophagy-induction function of Irgm1 is largely based on over-expression experiments using C-terminally EGFP-tagged Irgm1 [Gutierrez, 2004 #3064] [Singh, 2006 #3144]. However, we reported earlier that both N and C terminally tagged Irgm1 misbehave in cells [Martens, 2004 #3026]. To investigate to which compartments tagged Irgm1 localizes in cells, both N and C terminally EGFP-tagged Irgm1 were expressed in MEFs and co-stained with Golgi markers and endosomal/lysosomal markers (Figure 7). EGFP-Irgm1 and Irgm1-EGFP (pF25, [Gutierrez, 2004 #3064] [Singh, 2006 #3144] [Deghmane, 2007 #3610]) both showed diffuse vesicular and dotty expression pattern throughout the cytoplasm. Co-localization with the Golgi protein GM130 and TGN38 was abolished (Figure 7A, Fig 7B). Many of these vesicular and dotty signals strongly co localized with transferrin-labelled early endosomes and recycling endosomes (Figure 7C). As described earlier, no co-localization was found between native IFNγ-induced Irgm1 and early and recycling endosomes

(Figure 1C). The Irgm1-EGFP construct showed strong co-localization with LAMP1 while for EGFP-Irgm1 the lysosomal association was largely but not completely abolished (Figure 7D). Since Irgm1 associates with membranes independently of IFNγ-induced factors [Martens, 2004 #3026], we asked whether IFNγ could influence the mislocalization of EGFP-Irgm1 and Irgm1-EGFP. The two constructs were transfected into MEFs simultaneously induced with IFNγ. Both EGFP-Irgm1 and Irgm1-EGFP mislocalized also in IFNγ-induced cells (data not shown). In conclusion, neither N nor C terminally EGFP-tagged Irgm1 localizes to Golgi membranes, while both mislocalize to early and recycling endocytic compartments respectively.

Mislocalization of Irgm1 by EGFP tagging is nucleotide dependent.

Irgm1 is associated with Golgi membranes independently of nucleotide binding [Martens, 2004 #3026]. To test whether nucleotide binding influences the mislocalization of Irgm1 caused by EGFP tagging, the Irgm1 nucleotide binding deficient mutant (S90N) was tagged with EGFP at N or C terminus and expressed in the MEFs. Both fusion proteins accurately co-localized with Golgi protein TGN38 and GM130 (Figure 8 and not shown) but more cytoplasmic reticular and vesicular signals were also seen. The expression patterns of EGFP-Irgm1 (S90N) and Irgm1-EGFP (S90N) were similar to the localization of untagged nucleotide-binding deficient Irgm1 (S90N) [Martens, 2004 #3026].

Phagosomal accumulation of Irgm1 is nucleotide dependent, but IFNγ independent.

In L929 and Raw264.7 cells, IFNγ-induced Irgm1 is rapidly recruited to active plasma membrane upon phagocytosis and remains associated with phagosomes as they mature. It was also reported that Irgm1 associates with Golgi apparatus in an IFNγ and nucleotide independent manner [Martens, 2004 #3026]. We therefore asked whether Irgm1 can accumulate on phagosomes independently of IFNγ and if so, whether the association with phagosomes is regulated by nucleotide binding. Irgm1 wild type and

nucleotide binding deficient mutant S90N were expressed in MEFs in the absence of IFNγ and phagocytosis was initiated by incubating with 2-μm latex beads overnight. Wild type Irgm1 strongly associated with latex bead-phagosomes while Irgm1 S90N lost the phagosomal accumulation (Figure 9A). Transfected Irgm1 also co-localized with LAMP1 positive compartments in unstimulated cells taking up latex beads (Figure 9A arrows), as well as in cells not involved in phagocytosis (not shown). Irgm1 S90N still showed Golgi localization while lysosomal distribution was largely abolished (Figure 9A panels e-h). Therefore, the accumulation of Irgm1 on phagosomes is nucleotide dependent, but IFNγ independent.
As shown above, Irgm1-EGFP showed strong lysosomal association, while EGFP-Irgm1 did not. Consistent with this observation, Irgm1-EGFP accumulated strongly on latex bead phagosomes whereas EGFP-Irgm1 was absent or only very weakly present on phagosomes (Figure 9B). Like untagged Irgm1, the accumulation of Irgm1-EGFP on phagosomes is also regulated by nucleotide binding. Irgm1-EGFP S90N no longer associated with latex bead phagosomes (Figure 9C).

Discussion

The different members of the mouse IRG resistance protein family show different associations with cellular membranes. Unlike all the other IRGs, which have a more or less prominent cytosolic component, Irgm1 has been found exclusively in membrane-bound form in cells. Although no transmembrane domain could be identified in the sequences, Irgm1 behaves like a transmembrane protein in biochemical assays [Martens, 2004 #3026]. In L929 fibroblasts, TIB-75 hepatocytes and Raw 264.7 macrophages, Irgm1 localizes conspicuously to *cis*- and *medial*-Golgi apparatus with an additional cytoplasmic signal [Martens, 2004 #3026]. In the present study, by using TGN38 as a *trans*-Golgi and *trans*-Golgi network (TGN) marker, the Golgi localization domain of Irgm1 is extended to these compartments (Figure 1A). We also show that the additional cytoplasmic signals of Irgm1

overlap with those of LAMP1, which marks late endosomes and lysosomes. This observation is confirmed by co-localisation of Irgm1 with compartments enriched in the acidotropic dye LysoTracker (Figure 1B). We noted that, compared to the universal Golgi localization of Irgm1, the signal intensity of Irgm1 in the LAMP1-positive compartments varied in different individual cells, from conspicuous to hardly detectable. Since MEFs are isolated from mouse embryos and constitute a developmentally and morphologically heterogeneous population, the variable degrees of lysosomal association of Irgm1 could correlate with different states of individual cells, with stages of the cell cycle or with other undefined factors. Native endogenous Irgm1 is, however, scarcely if at all detectable on early and recycling endosomes defined by early and late uptake of fluorochrome-labelled transferrin (Figure 1C). Taken together, we show here for the first time that the IFNγ-induced endogenous Irgm1 localizes to the late endocytic/lysosomal compartments in addition to the Golgi apparatus. Irgm1 is the first IRG protein to be shown to localize to the endosomal/ lysosomal membrane system.

We further showed in MEFs that both N and C terminally EGFP-tagged Irgm1 (EGFP-Irgm1 and Irgm1-EGFP, respectively) localize differently from native Irgm1. Neither form of tagged protein localizes to the Golgi apparatus (Figure 7A and 7B). Irgm1-EGFP shows significant association with LAMP1-positive compartments in all transfected cells, in contrast to the variable lysosomal association of IFNγ-induced endogenous Irgm1 (Figure 7D). Unlike endogenous Irgm1, however, Irgm1-EGFP also localizes to early and recycling endosomes defined by the uptake of transferrin (Figure 7C). EGFP-Irgm1 associates with LAMP1-positive compartments very weakly, if at all and is distributed mainly in early and recycling endosomes as well as some other undefined, non-Golgi, non-LAMP1-positive, vesicular structures (Figure 7C and 7D). These observations have been confirmed on Irgm1 induced by IFNγ in Raw264.7 macrophages (unpublished). Deretic and colleagues reported that Irgm1-EGFP (pF25) co-localized with MDC and

LC3 positive compartments, suggesting that Irgm1 associates with autophagosomes and therefore has a pro-autophagy function in macrophages [Gutierrez, 2004 #3064] [Singh, 2006 #3144]. However, the present results raise the question whether an association of EGFP-tagged Irgm1 with autophagosomes may be only a secondary association from lysosomes, since Irgm1-EGFP associates constitutively with this compartments. Most importantly, Irgm1-EGFP behaves differently from endogenous IFNγ-induced Irgm1 in not localizing to the Golgi.

We found that both N and C terminally EGFP-tagged nucleotide-binding deficient Irgm1 (S90N) localized to the Golgi with little if any mislocalisation (Figure 8), indicating that the targeting of Irgm1 to different compartments is controlled by the nucleotide binding state of the protein. Golgi localization presumably reflects the inactive or GDP-bound state of the protein since nucleotide-binding deficient Irgm1 (S90N), whether tagged with EGFP or not, localizes to Golgi. Correspondingly, the endosomal and lysosomal localization of EGFP-tagged Irgm1 may reflect the activated or GTP-bound form of Irgm1, suggesting that the tags may cause constitutive or premature activation, or inhibit nucleotide exchange. We have recently shown that another IRG protein, Irga6, normally rests in cytoplasmic compartments in the inactive state, but depends on continuous regulatory interactions with Irgm1, Irgm2 and Irgm3 to inhibit spontaneous activation [Hunn, 2008 #3269]. IRG proteins belong to the dynamin-like large GTPase family, members of which show common properties of GTP-dependent oligomerisation and cooperative activity [Uthaiah, 2003 #3138]. We have shown that Irgm1 can interact with itself in a yeast 2-hybrid system, and self-interaction is abolished by destroying the nucleotide-binding motif (S90N) [Hunn, 2008 #3269]. Considering that the transfected EGFP proteins probably form dimers in cells [Yang, 1996 #3612], one possibility for the mislocalization of Irgm1 by an EGFP-tag could be that the interaction of two Irgm1 molecules is promoted by the EGFP dimer, thus prematurely stimulating the GTP-dependent activation of Irgm1.

Irgm1 deficient mice lose resistance to a broad range of intracellular pathogens, such as *T. gondii, T. cruzi, M. tuberculosis, S. typhimurium, Chlamydia trachomatis* and *L. monocytogenes* [Collazo, 2001 #2994] [MacMicking, 2003 #3010] [Feng, 2004 #3011] [Santiago, 2005 #3248] [Henry, 2007 #3512](Coers 2008) (Collazo 2001; MacMicking 2003; Santiago 2005; Henry 2007). Although those pathogens have diverse intracellular life styles, they share the property of establishing and residing, temporarily or permanently after entry into the host cells, in more or less modified vacuoles. So far, Irgm1 is the only member of the IRG family that has been found to associate with the endocytic, phagocytic and lysosomal compartments. Perhaps this distinctive property of Irgm1 correlates with its apparently pivotal role in resistance. MacMicking *et al* have suggested that Irgm1 may function by accelerating the acidification of *M. tuberculosis*-containing phagosomes, and Irgm1-deficient macrophages could not efficiently kill intracellular *M. tuberculosis* upon IFNγ treatment [MacMicking, 2003 #3010]. Furthermore, Irgm1 can accumulate on the *L. monocytogenes*-containing phagosomes [Shenoy, 2007 #3630] and escape from phagosomes by *L. monocytogenes* is efficiently blocked in IFNγ-treated macrophages [Portnoy, 1989 #3631]. How such a role for Irgm1 would relate to the similar role documented for Rab5a {Prada-Delgado, 2001 #3057} is unclear. However, the signal for Irgm1 to accumulate on phagosomes is not triggered only by pathogens, since phagocytosis of latex beads is sufficient to recruit Irgm1[Martens, 2004 #3026]. Furthermore any proposed direct action of Irgm1 on vacuolar pathogens cannot apply to the IRG-dependent destruction of the T. gondii parasitophorous vacuole membrane, since Irgm1 has been repeatedly found to be absent from this vacuole (Martens 2004, Butcher/Taylor, Hunn et al). Additionally, infection by T. gondii does not normally activate the phagocytic pathway [Morisaki, 1995 #3622] [Sibley, 2004 #3623] [Mordue, 1999 #3627]. It is of course plausible that Irgm1 may contribute to different mechanisms against different pathogens.

We showed recently that Irgm1, as well as its close relatives, Irgm2 and Irgm3, function primarily to regulate the activation of other "effector" members of the IRG protein family such as Irga6, Irgb6 and Irgd [Hunn, 2008 #3269]. There we argued that the regulatory interactions should occur primarily at cytoplasmic membranes rather than free in the cytosol. An obvious objection to that argument, however, was the apparent absence of a regulatory Irgm protein on the endo-lysosomal system. With the data of the present report, however, the endolysosomal system can now be included in the regulatory mechanism.

Golgi localization of Irgm1 is mediated by the predicted amphipathic αK helix near the C-terminus, a sequence that can also target EGFP to the Golgi [Martens, 2004 #3026]. We show here that the same sequence will also target EGFP to lysosomes defined by LAMP1. Oddly, about half the cells showed Golgi localization of EGFP-Irgm1 αK and the other half lysosomal localization. Only 14% of cells showed both. This may reflects in part the observation that IFNγ-induced endogenous Irgm1 shows different levels of lysosomal association, with strong lysosomal signals in some cells while with hardly detectable signals in other cells. The alternating behaviour of the EGFP-Irgm1 αK helix construct may be damped in the full-length protein due to the tethering effect of further membrane targeting signals ([Martens, 2004 #3026] and see below). The mechanism for this ambiguous, possibly dynamic localization behaviour is unclear: as noted above, it may correlate with spontaneous activation of the GTPase cycle.

The Irgm1 αK helix is required not only for targeting EGFP to Golgi and lysosomes, but also for the correct localization of full-length protein ([Martens, 2004 #3026] and this report). A mutant with glutamate insertions to disrupt the amphipathicity of αK helix in full-length Irgm1 distributes as uncharacteristic dotty structures throughout the cytoplasm, which show no overlap with GM130 or LAMP1 ([Martens, 2004 #3026] and Figure 2). The implication of this observation is that Golgi and lysosomal localization of full-length Irgm1 depends on the amphipathicity of the αK helix. However,

although Irgm1 αK helix is sufficient to target EGFP protein to Golgi and lysosomes, it is not sufficient to target EGFP to the latex bead phagosomes. Full-length Irgm1 is accumulated on phagosomes depending on the integrity of the nucleotide-binding motif. It seems likely that another conformationally active region of full-length Irgm1 may contribute to the phagosomal accumulation of the protein.

An alanine scan was performed to investigate the nature of the interaction of the EGFP-Irgm1 αK construct with Golgi or lysosomal membranes. Single mutations for each individual residue in the αK helical region and double/ quadruple mutations for hydrophobic residues did not change the Golgi/ lysosomal localization of EGFP-Irgm1 αK. The localization was also unaffected by single changes to charged or polar residues in the helical region. However, when more than two non-hydrophobic residues were substituted to alanines, EGFP-Irgm1 αK was distributed in an uncharacteristic reticular structure throughout the cytoplasm, overlapping neither with GM130 nor with LAMP1. These results suggest that both the overall amphipathicity of the αK helix and the identity of the non-hydrophobic residues contribute together to the membrane and Golgi/ lysosome localization of the αK helix. The insensitivity of the targeting specificity to exchanges of the hydrophobic residues to alanine suggests that the main function of these residues is to present a hydrophobic face and associate with the lipid component of the membrane. The contribution of the non-hydrophobic residues needs further analysis as it is unclear whether the effect observed from the double/triple non-hydrophobic-residue to alanine mutations is primarily due to an increase in overall hydrophobicity or to the loss of the specific polar side-chains. In addition, there are two basic residues (K349, R351) and three large hydrophobic residues (L350, L352, M353) preceding the predicted amphipathic helix that were not analyzed in the present study. Altogether there is a high frequency of basic residues in the αK targeting sequence, the positive charges associated with the sequence may be necessary to increase the overall affinity of the sequence for the

negatively charged head groups on the cytosolic face of the membrane. It is certainly not excluded that the αK targeting sequence may be targeted to bind to specific phosphoinositides, as suggested in a recent review [Shenoy, 2007 #3630].

Not only Irgm1, but all IRGM proteins have a membrane-targeting signal in the position of the αK helix in the Irga6 crystal structure. The αK helix from Irgm2 targets EGFP to the Golgi apparatus and the αK helix from Irgm3 targets EGFP to a reticular endomembrane system, both localizations that largely correspond to those of the respective full-length proteins [Martens, 2006 #3142]. Efforts to purify IRGM proteins for biochemical analysis have not been successful so far. We therefore took advantage of synthetic small targeting peptides to study the subcellular localization of IRGM proteins. The αK peptide from Irgm1 was a pioneer for this approach. There are several advantages to the peptide-streptavidin tetramer system. First, the targeting peptide from Irgm1 was insoluble in neutral pH buffers, but when loaded onto streptavidin protein, the solubility of the complex at neutral pH was adequate for experiment. Secondly, streptavidin is a natural tetrameric protein and each molecule has the capacity to bind four biotinylated peptides, resulting in enhanced avidity of the targeting complex to its target. Thirdly, streptavidin can be covalently labelled with fluorochromes and used directly in the fluoro-cytochemistry staining. Irgm1 αK peptide has been shown in the present study to successfully mimic the localization of endogenous full-length Irgm1 in both Golgi apparatus and lysosomal compartments in MEFs (Figure 6) and L929 cells (not shown). With synthetic targeting peptide-streptavidin complexes it would be relatively straightforward to analyse the putative phosphoinositide specificities of the targeting sequences recently suggested by Shenoy *et al* [Shenoy, 2007 #3630].

Materials and Methods

Expression constructs

EGFP-Irgm1 αK, EGFP-Irgm1, Irgm1-EGFP (pF25), pGW1H-Irgm1, pGW1H-Irgm1 S90N, pGW1H-Irgm1 ins 362, 367E constructs were generated as described[Martens, 2004 #3026] [Gutierrez, 2004 #3064]. Site directed mutagenesis was performed by the Quik-Change Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA).

Cells and tissue culture.

C57BL/6 embryonic fibroblasts (MEFs) were prepared from mice at day 14 *post coitum* and cultured in DMEM (high glucose) (Invitrogen, Carlsbad, CA) supplemented with 10% FCS (Biochrom AG, Berlin, Germany), 2mM L-glutamine, 1mM sodium pyruvate, non-essential amino acids, 100 U/ml penicillin, 100 mg/ml streptomycin (all PAA, Pasching, Austria). Cells were transiently transfected using FuGENE6 (Roche, Basel, Switzerland) according to the manufacturer's instructions. Mouse IFNγ was purchased from PeproTech, NJ, USA.

Transferrin uptaking experiments

Cells were grown on coverslips and starved for 1 hour in FCS-free medium. To label early endosomes, Alexa-546 labeled human Transferrin (Molecular Probes, Carlsbad, CA) was diluted to a final concentration of 5 μg/ml into the FCS-free medium, and cells were incubated at 37°C, 7.5% CO_2 for 5 minutes. Cells were then fixed with ice-cold 3% PFA in PBS for 20 minutes. To label recycling endosomes, the cells were incubated with FCS-free medium with diluted transferrin for 10 minutes after starvation, then washed 3 times with ice-cold PBS. Complete medium was added afterwards and the cells were incubated at 37°C, 7.5% CO_2 for 30 minutes. Finally cells were fixed with ice-cold 3% PFA in PBS for 20 minutes followed by three washes with PBS and stained for immunofluorescence.

Lysotracker loading experiments

Lysotracker Red DND-99 (Molecular Probes) was diluted to a final concentration of 50 nM in complete medium and the cells were incubated at

37°C, 7.5% CO_2 for 20 minutes. Cells were then fixed with ice-cold 3% PFA in PBS for 20 minutes followed by three washes with PBS and stained for immunofluorescence.

Latex bead phagocytosis experiments

MEFs were grown on the coverslips, treated with 200 U/ml IFNγ and/or transfected with indicated constructs for 24 hours. During the IFNγ-treatment and/or transfection procedure, 2 μm carboxylated latex beads (Polysciences, Warrington, PA) were added to the culture at a dilution of 1:1000, and the cells were incubated with the latex beads at 37°C overnight. The latex beads were extensively phagocytosed by the MEFs through unidentified receptors. Finally the cells were fixed with 3% PFA for 20 minutes at room temperature and stained for immunofluorescence.

Peptide-streptavidin complexes

The Irgm1 αK peptide H-SK(Biotin)LRLMTCAIVNAFFRLLRFLPCVCC-OH was synthesized by JPT Peptide Technologies GmbH, Berlin.

The peptide was biotinylated on Lysine at the second position of the sequence. The peptide was dissolved in 10mM NaAc pH4.5 buffer with 10mM TCEP as reducing agent and stored at -80°C at a stock concentration of ~100 μM. The absolute absorbance of the Irgm1 αK peptide at 230 nm in the dissolving buffer was ~0.46/100 μM.

The peptide-Cy3-streptavidin complex was made in the blocking buffer used for the immunofluorescence staining (PBS pH7.4/0.1% saponin/1% BSA with additional 2 mM DTT). The Cy3-labelled streptavidin (Sigma, S6402) was first diluted in the blocking buffer to a final concentration of 20 μg/ml (0.33 μM). The peptide stock solution was then added to the streptavidin solution with a titration from 4:1 to 50:1 molar ratio (peptide to streptavidin). The optimum ratio was experimentally determined based on the staining signals (8 μM peptide in the Figure 6). The peptide-streptavidin solution was mixed and precipitated material removed by centrifugation at 45.000 rpm for 30 minutes. The supernatant was recovered and antibodies against the indicated proteins

added. This solution was used like a primary antibody in immunofluorescence staining, and was incubated with PFA-fixed saponin permeabilized cells at 37°C for 1 hour. The peptide-streptavidin images were obtained in the Cy3 channel.

Immunofluorescent staining

Immunofluorescent staining was performed on paraformaldehyde-fixed cells essentially as described earlier [Martens, 2004 #3026]. Images were taken with a Zeiss Axioplan II fluorescence microscope equipped with an AxioCam MRm camera (Zeiss) and processed with Axiovision 4.6 software (Zeiss). 4',6-Diamidine-2'-phenylindole dihydrochloride (DAPI, Invitrogen) was used for nuclear counterstaining at a final concentration of 0.5 μg/ml.

Immunoreagents

The following serological reagents were used for immunofluorescence at the indicated concentrations: anti-Irgm1 goat polyclonal antibody P20 (1:100, Santa Cruz Biotechnology), anti-Irgm1 mouse monoclonal antibody 1B2 (supernatant recovered from hybridoma cell culture [Butcher, 2005 #3096]), anti-Irgm2 rabbit antiserum H53 (1:1000, [Martens, 2005 #3082]), anti-GM130 mouse monoclonal antibody (1:1000, BD Transduction Lab, 610822), anti-TGN38 goat polyclonal antibody S-20 (1:100, Santa Cruz Biotechnology), anti-CI-M6PR rabbit antiserum (1:100, a gift from Albert Haas, University of Bonn), anti-LAMP1 rat monoclonal antibody 1D4B (1:1000, University of Iowa, USA), goat anti-mouse Alexa 488 and 546, goat anti-rabbit Alexa 488 and 546, donkey anti-rat Alexa 488, donkey anti-goat Alexa 350, 488, 546 and 647, donkey anti-mouse Alexa 488, 555 and 647, donkey anti-rabbit Alexa 488, 555 and 647 (1:1000, Molecular Probes, Invitrogen).

Figure legends

Figure 1. Localization of IFNγ-induced endogenous Irgm1.

(A) MEFs were treated with 200U/ml IFNγ for 24 hours, fixed and stained for Irgm1 with goat antiserum P20 (a-c, g-l) or mouse monoclonal antibody 1B2 (d-

f) (both directed against Irgm1) and the indicated marker proteins. Irgm1 accurately co-localized with GM130 (a-c) and TGN38 (d-e), and localized adjacent to, or partially co-localized with, the CI-M6PR positive compartments (g-i). (B) MEFs were treated with 200U/ml IFNγ for 24 hours. Cells were either fixed and stained for Irgm1, LAMP1 and Irgm2 using 1B2, 1D4B, and H53 immunoreagents, respectively (a-d), or further incubated with 50 nM LysoTracker Red DND-99 for 30 minutes in complete medium, and stained for Irgm1 with 1B2 antibody (e-g). Irgm1 was found to associate with LAMP1 positive compartments (a-d) and accumulate around the LysoTracker enriched compartments (e-g) in addition to Golgi localization. Irgm2 (c) localizes exclusively to the Golgi apparatus. (C) After treatment with 200U/ml IFNγ for 24 hours, MEFs were incubated with Alexa-Fluor-546-labelled transferrin for 5 minutes (a-c) or pulsed with labelled transferrin for 10 minutes, then chased for 30 minutes (d-f). Cells were then fixed and stained for Irgm1 with P20 goat antiserum. The granular signals from Irgm1 were not found associated with transferrin-positive compartments.

Figure 2. Amphipathic αK helix is responsible for lysosomal targeting of full length Irgm1 protein.

MEFs were transfected with plasmids encoding either Irgm1 wild type (a-c) or the ins 362, 367E mutant (d-f) for 24 hours in the absence of IFNγ. Cells were then fixed and stained for Irgm1 and LAMP1. Wild type Irgm1 is strongly associated with the LAMP1 positive compartment, while Irgm1 ins 362, 367E mutant showed granular signals throughout cytoplasm which do not overlap with LAMP1 signals. Nuclei were labelled with DAPI.

Figure 3. EGFP-Irgm1 αK localizes to both Golgi and lysosomes.

MEFs were transfected with an expression plasmid encoding EGFP-Irgm1 αK in the absence of IFNγ (). 24 hours later cells were fixed and stained for GM130 (a-i) and LAMP1 (j-o). 32% transfected cells showed only Golgi localization (a-c) and 54% showed only dotty structures among which strong LAMP1 co-localization was observed (g-l). Expression in 14% transfected cells showed

both Golgi and LAMP1 co-localization (d-f and m-o). Nuclei were labelled with DAPI.

Figure 4. EGFP-Irgm1 αK is not recruited to phagosomes.

EGFP-Irgm1 αK was transiently transfected into MEFs for 24 hours in the absence of IFNγ. During transfection, cells were incubated with 2-μm latex beads overnight. Cells were then fixed and stained for LAMP1. EGFP-Irgm1 αK was not associated with bead phagosomes, whether the association with other cellular compartments was Golgi only (a-d), lysosomal only (e-h) or both Golgi and lysosomal (i-l). Despite not associating with latex bead phagosomes, EGFP-Irgm1 αK associated normally with the LAMP1 compartment elsewhere (arrows). Nuclei were labelled with DAPI.

Figure 5. Alanine mutagenesis scan of EGFP-Irgm1 αK.

EGFP-Irgm1 αK quadruple mutant (F362A F363A L365A L366A; Figure 6A), single mutants of the hydrophilic residues (N360A；R364A；R367A; Figure 6B including enlargements (for d, e, f)), or combined mutants of more than one hydrophilic residues (N360A, R364A; R364A, R367A; N360A, R364A, R367A; Figure 6C) were transfected into MEFs for 24 hours. Cells were then fixed and staining for GM130 or LAMP1. Only when more than one hydrophilic residue was mutated to alanine, the EGFP-Irgm1 α K no longer localized to Golgi or lysosomes. These mutants localized to unidentified endomembrane-like structures. Nuclei were labelled with DAPI.

Figure 6. Synthetic αK amphipathic peptide mimics the localization of endogenous Irgm1.

MEFs were treated with 200U/ml IFNγ for 24 hours and then fixed with 3% PFA in PBS. Cy3-streptavidin-peptide solutions (0.33μM streptavidin) were prepared as described in Materials and Methods. Antibodies against the indicated proteins were added to the streptavidin-peptide solution and the mixtures used as primary reagents in immunofluorescence staining. Streptavidin (0.33μM final) alone (a) was used as control. Image (a) and (b) were taken at the

same exposure time. Colocalization was seen between streptavidin-Irgm1 αK peptide and endogenous Irgm1 at both Golgi apparatus and lysosomes.

Figure 7. Localization of EGFP-tagged Irgm1.

(A) N-terminally EGFP-tagged Irgm1 (EGFP-Irgm1) or (B) C-terminally EGFP-tagged Irgm1 (Irgm1-EGFP, pF25) were transfected into MEFs. 24 hours later, cells were fixed and stained for GM130 (a-c) and TGN38 (d-f). Both constructs were absent from the Golgi apparatus. Nuclei were labelled with DAPI. (C) EGFP-Irgm1 (a-f) or Irgm1-EGFP (pF25, g-l) were transfected into MEFs. 24 hours later cells were incubated with Alexa-Fluor-546-labelled transferrin for 5 minutes (a-c, g-i) or pulsed with labelled transferrin for 10 minutes, then chased for 30 minutes (d-f, j-l). Cells were then fixed in 3% PFA in PBS. The vesicular and dotty signals from EGFP-Irgm1 and Irgm1-EGFP strongly overlap with transferrin-labelled early and recycling endosomes. (D) EGFP-Irgm1 (a-c) or Irgm1-EGFP (d-f, pF25) were transfected into MEFs. 24 hours later, cells were fixed and stained for LAMP1. Irgm1-EGFP was strongly colocalized with LAMP1 while lysosomal association of EGFP-Irgm1 was largely but not completely abolished. Nuclei were labelled with DAPI.

Figure 8. Mislocalization of EGFP-tagged Irgm1 is nucleotide-dependent.

EGFP-Irgm1 S90N (a-c) and Irgm1-EGFP S90N (d-f) were transfected into MEFs for 24 hours. Cells were then fixed and stained for TGN38. Both constructs co-localized with TGN38, although a significant proportion of transfected proteins displayed additional cytoplasmic signals. Nuclei were labelled with DAPI.

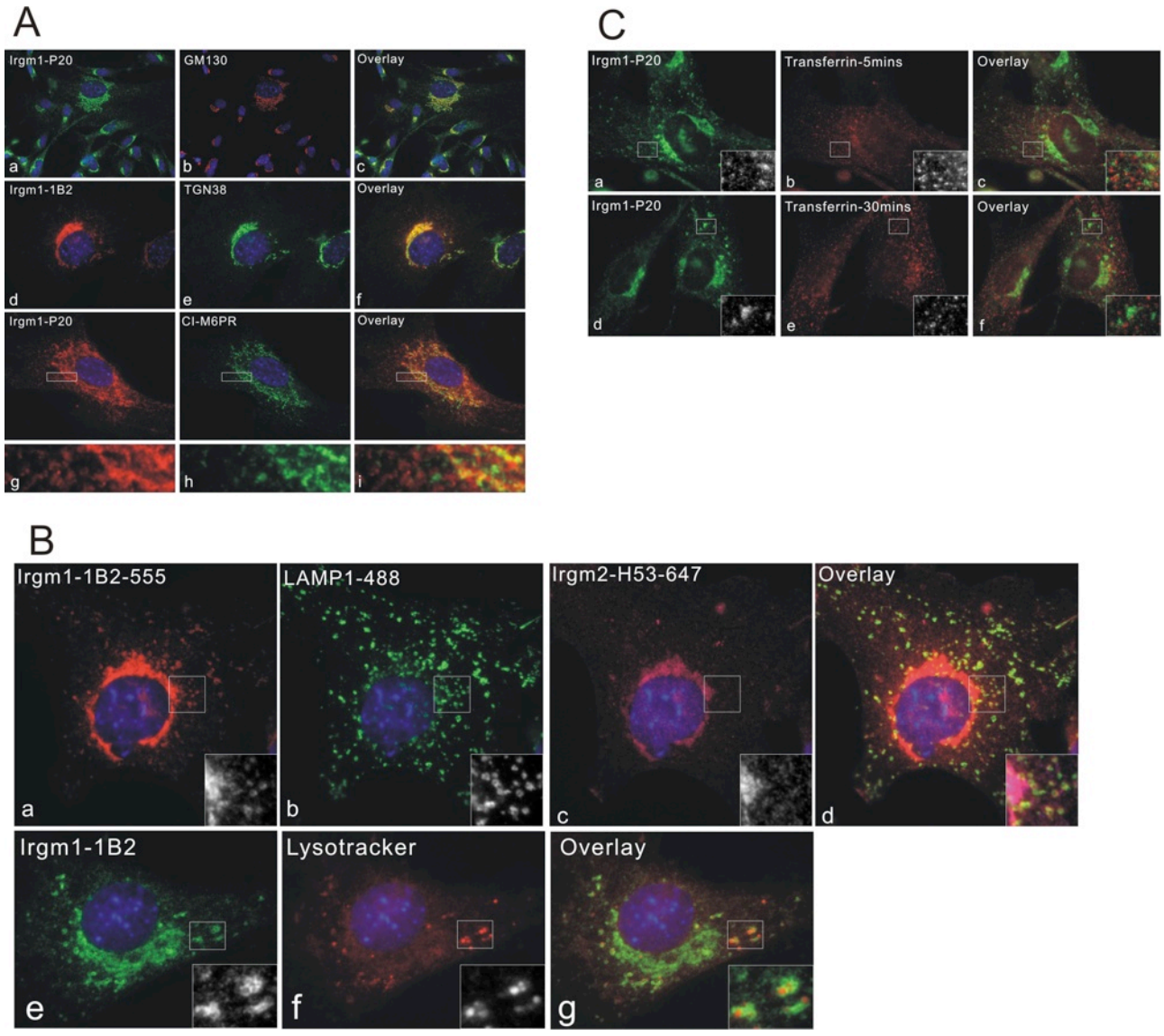
Figure 9. Phagosomal accumulation of Irgm1 is nucleotide-dependent, but IFNγ-independent.

(A) Irgm1 wild type (a-d) and S90N mutant (e-h) were transfected into MEFs in the absence of IFNγ for 24 hours. During transfection, cells were incubated with 2-μm latex beads overnight. Cells were then fixed and stained for Irgm1 and

LAMP1. Irgm1 accumulated around the latex beads phagosomes while the loss of the nucleotide binding site completely abolished the phagosomal accumulation. Arrows indicate the co-localization of transfected wild type Irgm1 with LAMP1 positive compartments outside latex-bead phagosomes. (B) EGFP-Irgm1 or Irgm1-EGFP were transfected into MEFs for 24 hours in the absence of IFNγ. During transfection, cells were also incubated with 2-μm latex beads overnight. Cells were then fixed and stained for LAMP1. Irgm1-EGFP accumulated around the latex bead phagosomes while EGFP-Irgm1 did not. (C) The same experiments were performed as in (B) but using the nucleotide-binding deficient mutants (S90N) of N- and C-terminally EGFP-tagged Irgm1. Both mutants were absent from latex bead phagosomes. Nuclei were labelled with DAPI.

References

Fig 1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CI-M6PR
Overlay
g
h
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C
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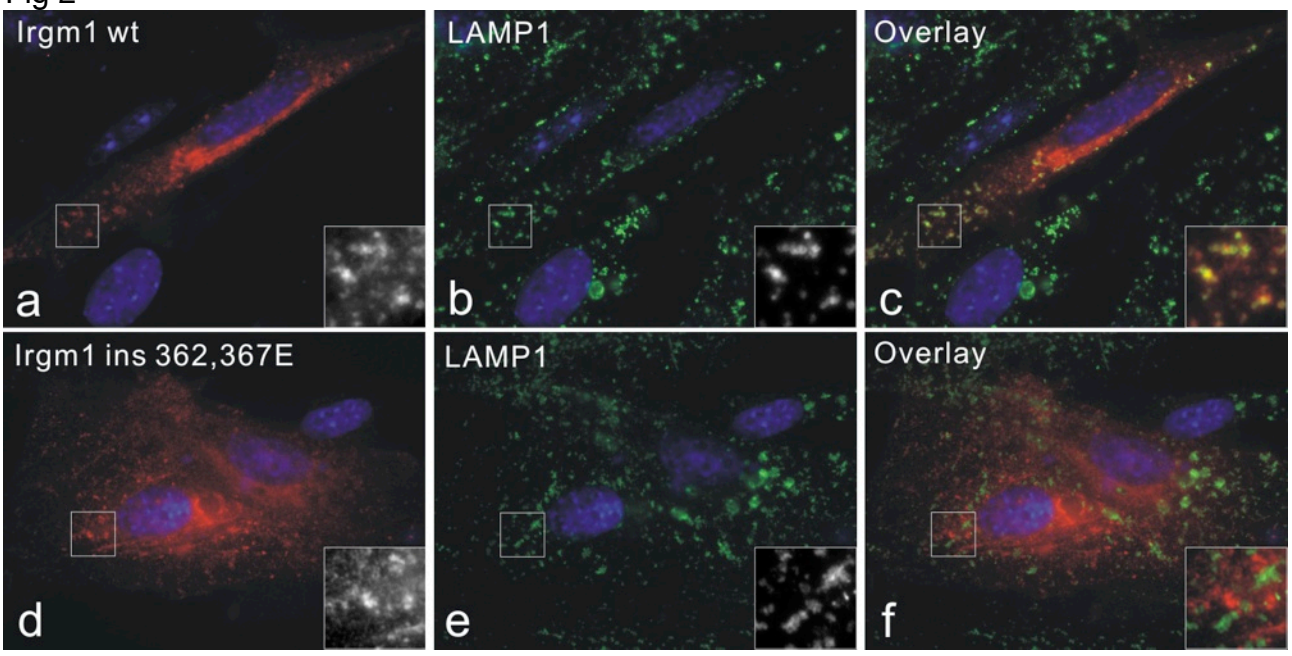
g

Fig 2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d
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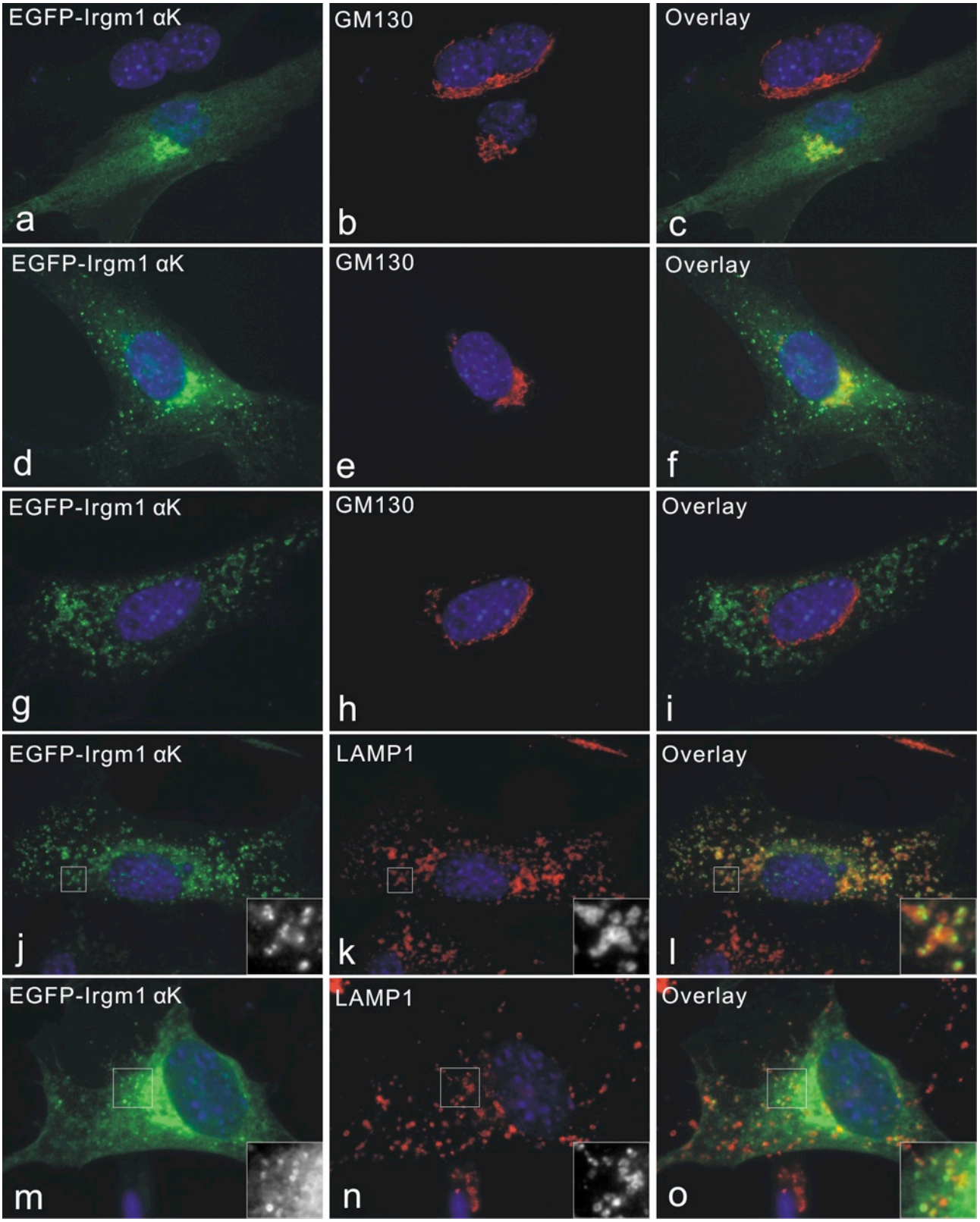
f

Fig 3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g
h
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m
n
o

Fig 4

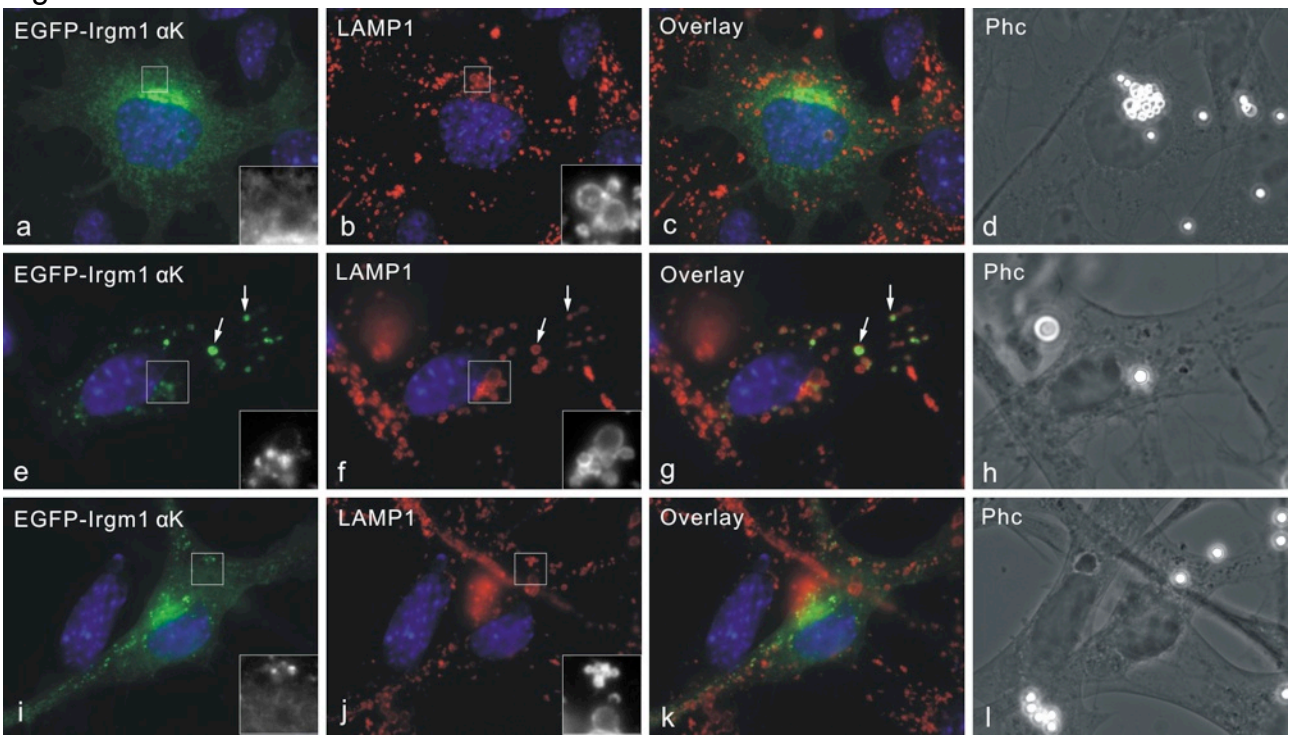
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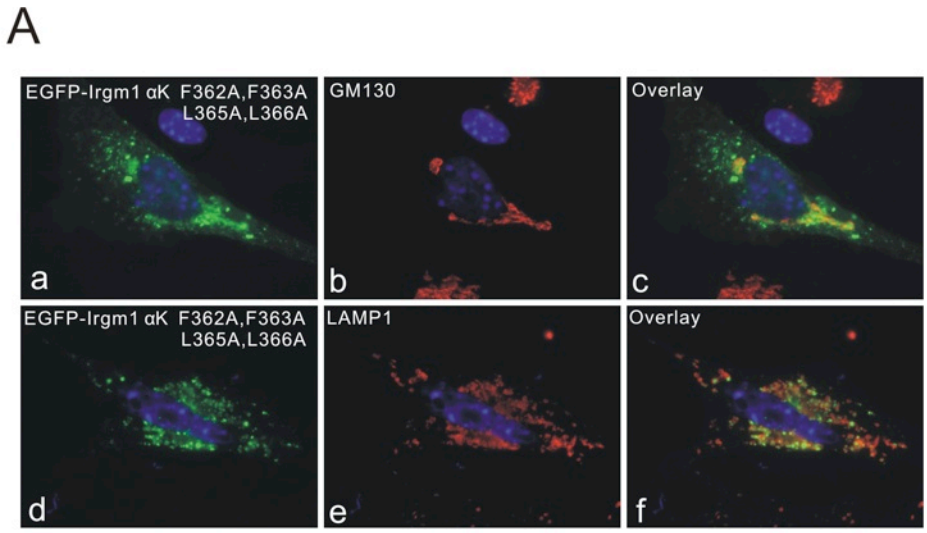
Fig 5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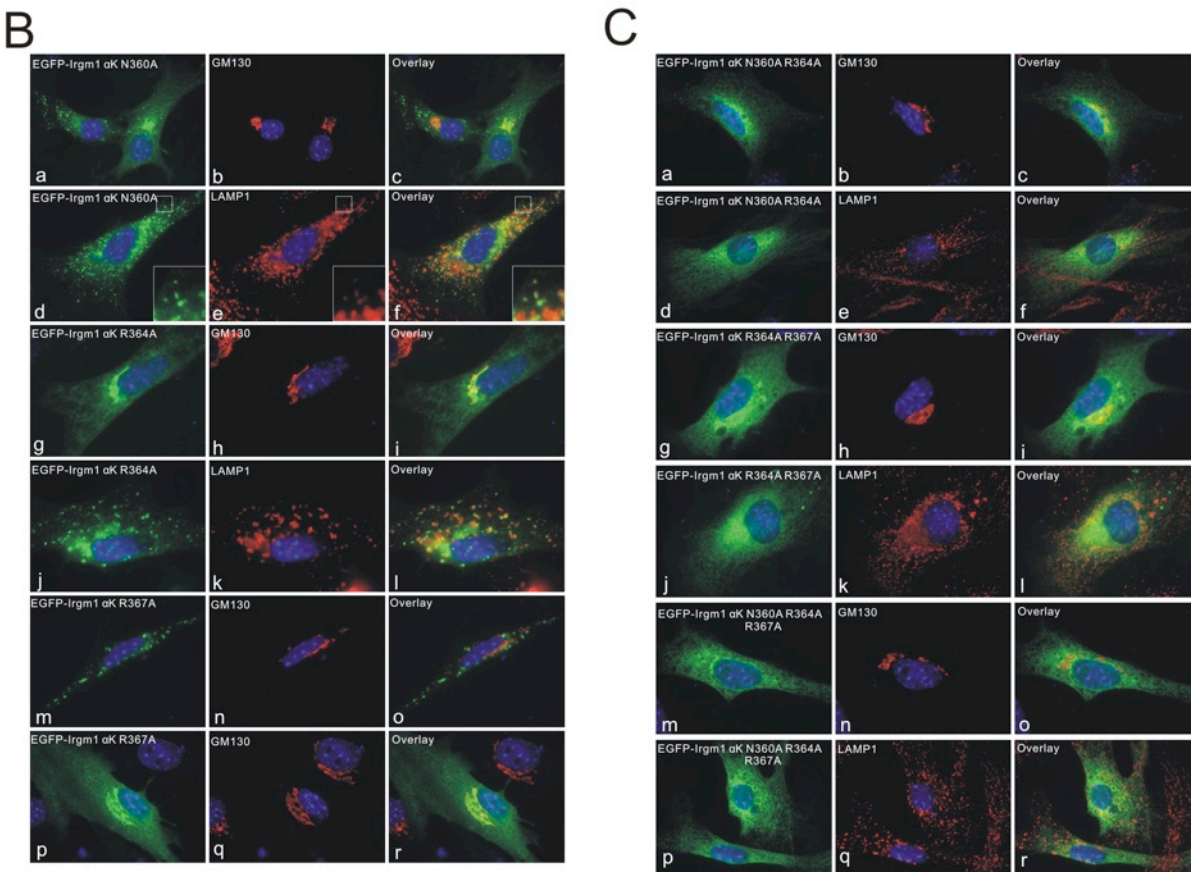
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Fig 6

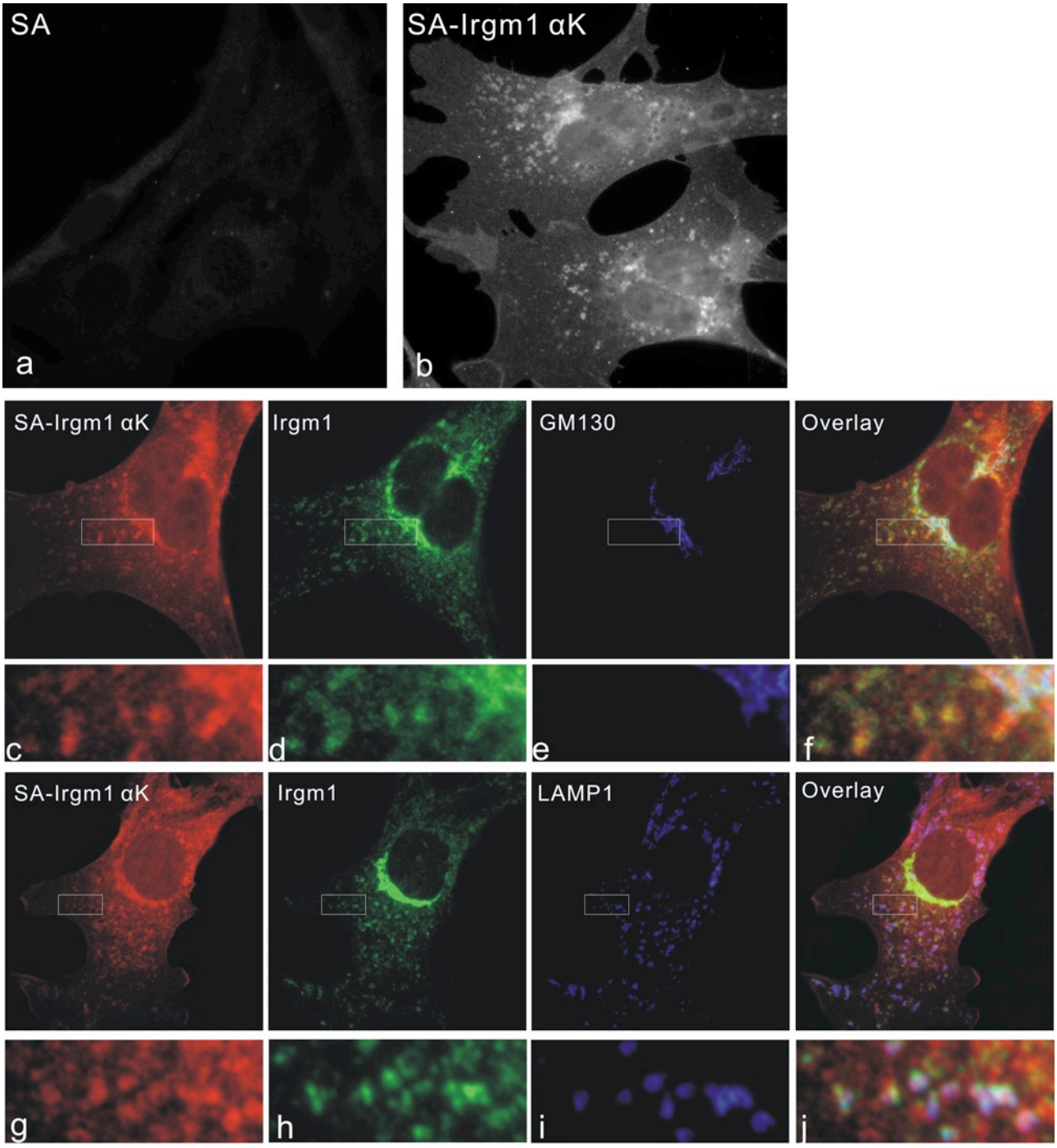
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Fig 7

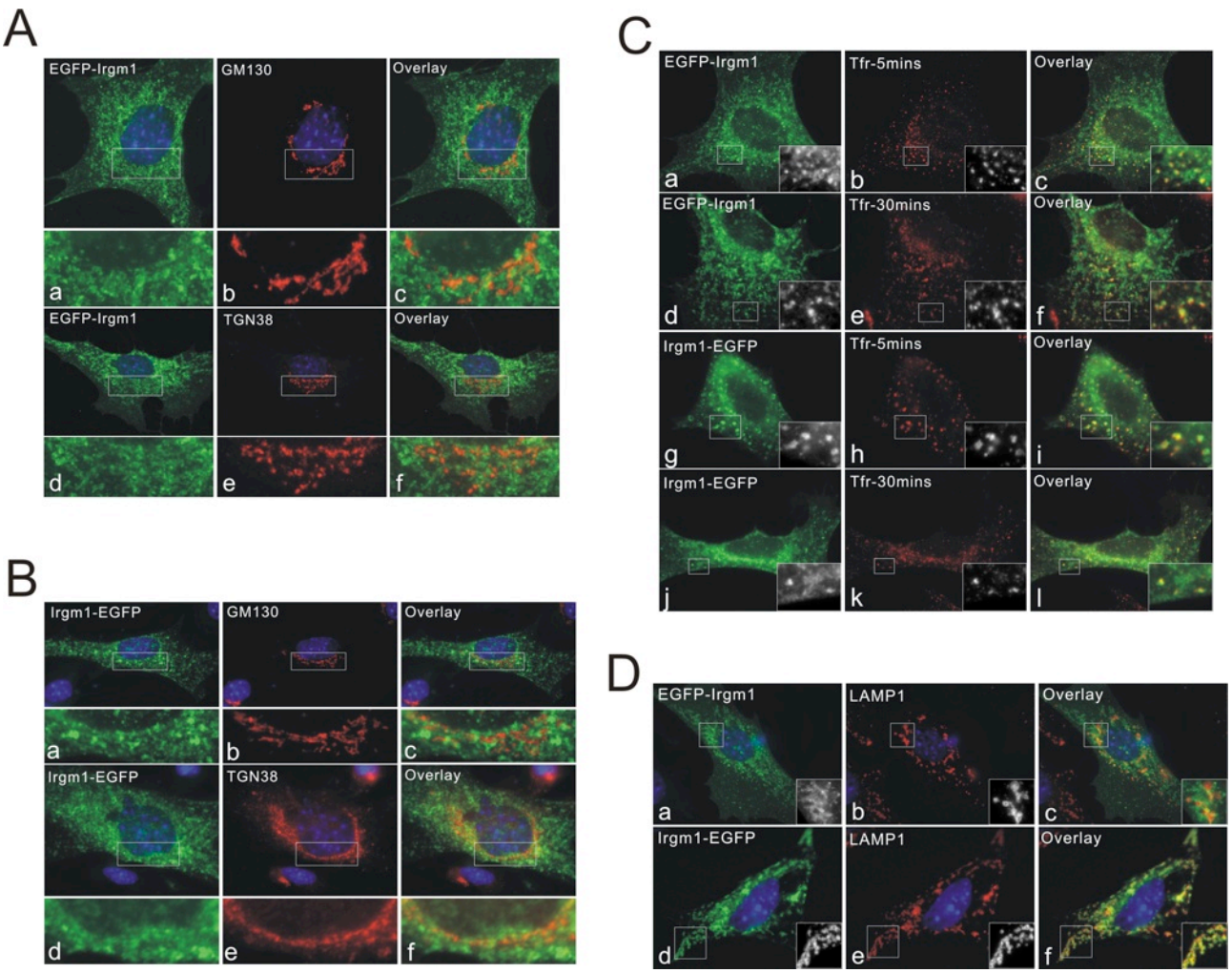
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Fig 8

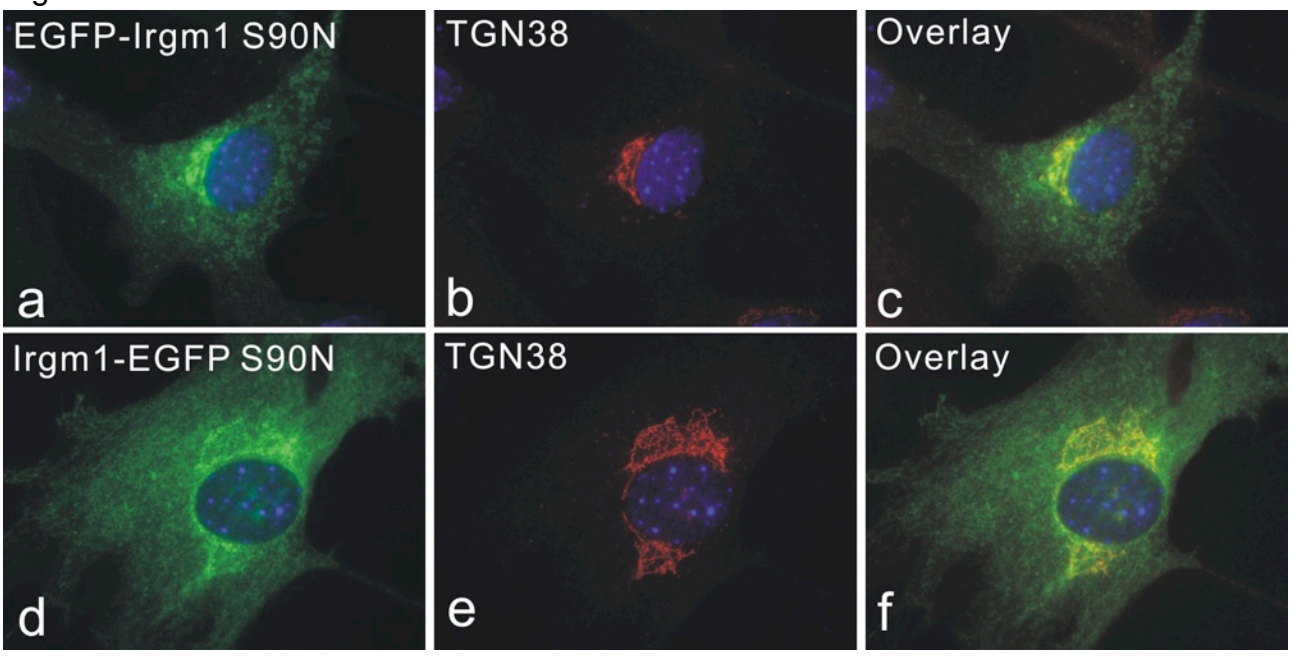
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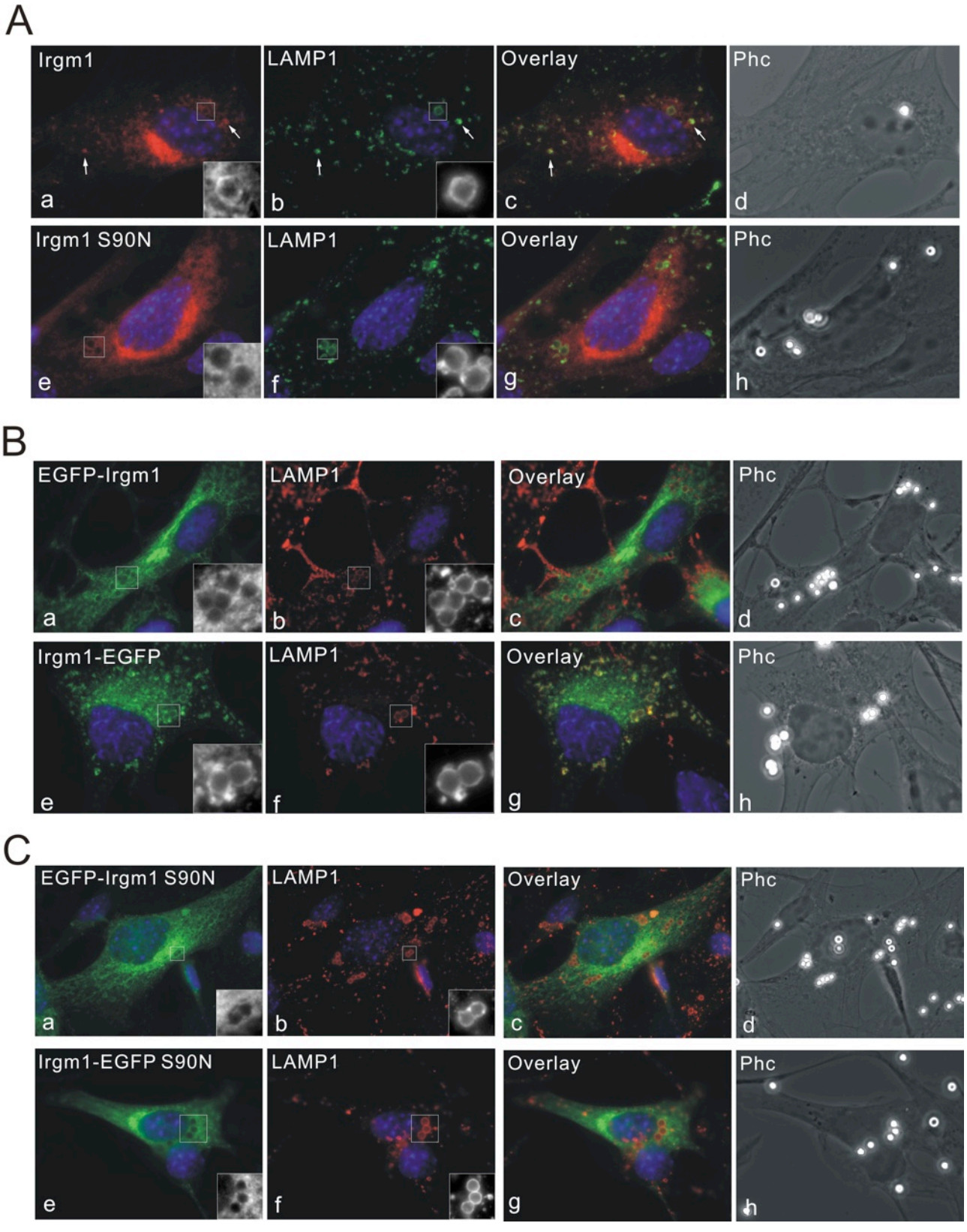
Fig 9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