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**Galectin-8 senses phagosomal damage and recruits selective autophagy  
adapter TAX1BP1 to control *Mycobacterium tuberculosis* infection in  
macrophages**

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Keywords: xenophagy, bacterial pathogenesis

23 **ABSTRACT**

24 *Mycobacterium tuberculosis* (Mtb) infects a quarter of the world and causes the deadliest infectious  
25 disease worldwide. Upon infection, Mtb is phagocytosed by macrophages and uses its virulence-  
26 associated ESX-1 secretion system to modulate the host cell and establish a replicative niche. We have  
27 previously shown the ESX-1 secretion system permeabilizes the Mtb-containing phagosome and that a  
28 population (~30%) of intracellular Mtb are recognized within the cytosol, tagged with ubiquitin, and  
29 targeted to the selective autophagy pathway. Despite the importance of selective autophagy in controlling  
30 infection, the mechanisms through which macrophages sense and respond to damaged Mtb-containing  
31 phagosomes remains unclear. Here, we demonstrate that several cytosolic glycan-binding proteins,  
32 known as galectins, recognize Mtb-containing phagosomes. We found that galectins-3, -8, and -9 are all  
33 recruited to the same Mtb population that colocalizes with selective autophagy markers like ubiquitin,  
34 p62, and LC3, which indicates Mtb damages its phagosomal membrane such that cytosolic host sensors  
35 can recognize danger signals in the lumen. To determine which galectins are required for controlling Mtb  
36 replication in macrophages, we generated CRISPR/Cas9 knockout macrophages lacking individual or  
37 multiple galectins and found that galectin-8<sup>-/-</sup> and galectin-3/8/9<sup>-/-</sup> knockout macrophages were similarly  
38 defective in targeting Mtb to selective autophagy and controlling replication, suggesting galectin-8 plays  
39 a privileged role in anti-Mtb autophagy. In investigating this specificity, we identified a novel and specific  
40 interaction between galectin-8 and TAX1BP1, one of several autophagy adaptors that bridges cargo and  
41 LC3 during the course of autophagosome formation, and this galectin-8/TAX1BP1 interaction was  
42 necessary to efficiently target Mtb to selective autophagy. Remarkably, overexpressing individual  
43 galectins increased targeting of Mtb to antibacterial autophagy and limited Mtb replication. Taken  
44 together, these data imply that galectins recognize damaged Mtb-containing phagosomes, recruit  
45 downstream autophagy machinery, and may represent promising targets for host-directed therapeutics  
46 to treat Mtb.

47

48

## 49 INTRODUCTION

50 *Mycobacterium tuberculosis* (Mtb), which causes tuberculosis, infects approximately 10 million  
51 people annually and kills about 1.5 million, making it the deadliest infectious disease worldwide (World  
52 Health Organization, 2019). Spread in aerosolized droplets when an infected person coughs, Mtb travels  
53 to the depths of the lungs where it is phagocytosed by alveolar macrophages. Typically, macrophages  
54 are incredibly efficient at identifying and destroying invading microbes, and they have numerous potent  
55 killing mechanisms, including lysosomal degradation, reactive oxygen species, antimicrobial peptides,  
56 guanylate-binding proteins (GBPs), and autophagy (Weiss & Schaible, 2015). However, Mtb employs  
57 strategies to resist nearly all of these defense mechanisms and survives and replicates in macrophages  
58 (Kaufmann & Dorhoi, 2016; Upadhyay et al., 2018). Understanding the few mechanisms by which  
59 macrophages can successfully control Mtb is critical for the future development of effective therapies for  
60 this difficult-to-treat pathogen.

61 One way a macrophage can control intracellular Mtb is through selective autophagy, a specific  
62 form of autophagy whereby a cell tags unwanted cytosolic cargo with ubiquitin, which serves as an “eat  
63 me” signal (Boyle & Randow, 2013; Khaminets et al., 2016; Stolz et al., 2014). Ubiquitin-tagged cargo  
64 can then be coated by a variety of selective autophagy adapters (p62/SQSTM1, Calcoco2/NDP52,  
65 Optineurin/OPTN, etc.), which have ubiquitin-binding domains that promote their recruitment to tagged  
66 cargo. These adapters also have an LC3 interaction region (LIR), a motif that enables binding to the  
67 autophagy protein LC3 and the closely related GABARAP proteins (Wild et al., 2014). As a result,  
68 selective autophagy adapters serve as bridges between ubiquitinated cargo and the LC3-decorated  
69 autophagophore that will ultimately engulf and degrade the cargo. Numerous types of cargo, including  
70 damaged mitochondria (mitophagy), protein aggregates (aggrephagy), and cytosolic pathogens  
71 (xenophagy), can be degraded via selective autophagy, and various subsets of adapters are associated  
72 with different types of cargo. For example, mitophagy utilizes NDP52 and OPTN, aggrephagy p62 and  
73 NBR1, and xenophagy p62 and NDP52 (Farré & Subramani, 2016; Stolz et al., 2014). However, the  
74 biology underlying the redundancy and specificity of these adapters remains poorly understood.

75           Several lines of evidence indicate that selective autophagy is required for controlling Mtb infection.  
76 Our work and that of others have shown that ESX-1-dependent permeabilization of the Mtb phagosome  
77 allows the cytosolic DNA sensor cGAS to detect bacterial dsDNA, which triggers both a pro-bacterial type  
78 I interferon (IFN) transcriptional response (via the STING/TBK1/IRF3 signaling axis) and anti-bacterial  
79 selective autophagy (Collins et al., 2015; Manzanillo et al., 2012; Wassermann et al., 2015; Watson et  
80 al., 2012, 2015). Specifically, within 4-6 h after infection, approximately 30% of intracellular Mtb bacilli  
81 are surrounded by ubiquitin, LC3, and several selective autophagy adapters (Watson et al., 2012). In the  
82 absence of selective autophagy targeting (i.e., adapter-deficient macrophages), Mtb survives and  
83 replicates to a higher degree (Watson et al., 2012). While the precise nature of the ubiquitination of Mtb  
84 is unclear, several E3 ligases, including Parkin, Smurf1, and TRIM16 colocalize with a subset of Mtb  
85 phagosomes and are required for optimal tagging of Mtb with ubiquitin (Chauhan et al., 2016; Franco et  
86 al., 2017; Manzanillo et al., 2013). These E3 ligases are required for controlling Mtb replication in  
87 macrophages, and Parkin and Smurf1 are further required for controlling Mtb infection *in vivo* in mouse  
88 models of infection. Likewise, macrophages lacking the core autophagy protein ATG5 fail to control Mtb  
89 replication, and mice with a macrophage-specific ATG5 deletion are incredibly sensitive to Mtb infection  
90 and succumb within weeks (Watson et al., 2012). A subsequent report found that ATG5 plays a critical  
91 role in neutrophil-mediated inflammation, suggesting autophagy functions in both cell-intrinsic and cell-  
92 extrinsic immune responses (Kimmey et al., 2015).

93           We are continuing to understand the function, impact, and scope of selective autophagy in  
94 controlling Mtb infection, and the precise mechanisms used by macrophages to detect damaged Mtb  
95 phagosomes and intracellular Mtb bacilli are remain poorly defined. Our previous studies have found that  
96 cytosolic DNA sensing through cGAS/STING/TBK1 is required for recognition and targeting;  
97 macrophages lacking cGAS or STING target half as many Mtb bacilli to selective autophagy (Watson et  
98 al., 2015). However, because a sizable population of Mtb are targeted even in the absence of DNA  
99 sensing, it is likely that additional “danger signals” (e.g., microbes or damage caused by microbes) and  
100 “danger sensors” are employed by macrophages during Mtb infection (Vance et al., 2009).

101 One class of danger sensors are galectins, which are a large, highly conserved family of proteins  
102 that bind to glycosylated proteins and lipids via their carbohydrate recognition domains (CDRs)  
103 (Rabinovich & Toscano, 2009; van Kooyk & Rabinovich, 2008; Vasta, 2009). Despite having no classical  
104 secretion signal, many galectins are extracellular where they can bind to glycosylated proteins and lipids  
105 on cell surfaces or in the extracellular matrix to modulate cellular processes like signaling, adherence,  
106 and migration (Rabinovich & Toscano, 2009; van Kooyk & Rabinovich, 2008). Several galectins are also  
107 found in the cytosol where they exert other functions, including acting as soluble receptors for endosomal  
108 or lysosomal membrane damage. After disruption of membranes, galectins can access and bind to  
109 glycans within the lumen of damaged membrane-bound compartments (Boyle & Randow, 2013;  
110 Khaminets et al., 2016; Thurston et al., 2012). Often, intracellular bacteria inflict this type of endosomal  
111 damage, and galectins-3, -8, and -9 have been found to colocalize with several intracellular pathogens,  
112 including *Salmonella* Typhimurium, *Shigella flexneri*, *Listeria monocytogenes*, *Legionella pneumophila*,  
113 and *Yersinia pseudotuberculosis* (Feeley et al., 2017; Thurston et al., 2012). In some cases, the  
114 functional consequences of galectin recruitment to intracellular bacteria have been characterized. During  
115 *L. pneumophila* and *Y. pseudotuberculosis* infection, galectin-3 promotes the recruitment of antibacterial  
116 GBPs to bacteria, and during *S. Typhimurium* infection of HeLa cells, galectin-8 recruits NDP52, which  
117 brings autophagy machinery to exposed bacteria. While some of these pathways have been studied in  
118 detail, the specific molecular mechanisms by which macrophages use galectins to detect and target Mtb  
119 to selective autophagy have not been fully characterized.

120 Here we show that galectins-3, -8, and -9 are recruited to Mtb in macrophages, and that galectin+  
121 bacteria are the same population targeted to selective autophagy. Deletion of galectin-8, but not  
122 galectins-3 or -9, decreased targeting of Mtb as monitored by LC3 recruitment and by bacterial  
123 survival/replication. Deleting all three galectins did not amplify these phenotypes, suggesting galectin-8  
124 is the most crucial for recognition and targeting of Mtb in macrophages. Using immunoprecipitation and  
125 mass spectrometry, we found that galectin-8 interacts with the selective autophagy adapter TAX1BP1,  
126 but this interaction is independent of TAX1BP1's ubiquitin-binding domain. Furthermore, in Mtb-infected

127 macrophages, we found that the recruitment of TAX1BP1 to Mtb required both its interaction with galectin-  
128 8 and its ubiquitin-binding domain. Finally, we found that overexpression of galectins-8 and -9 significantly  
129 augmented the ability of macrophages to control Mtb survival and replication, indicating that while specific  
130 galectins may not be essential for targeting Mtb to selective autophagy, they are sufficient, which raises  
131 the possibility of targeting this detection and destruction pathway for the development of future host-  
132 directed therapies.

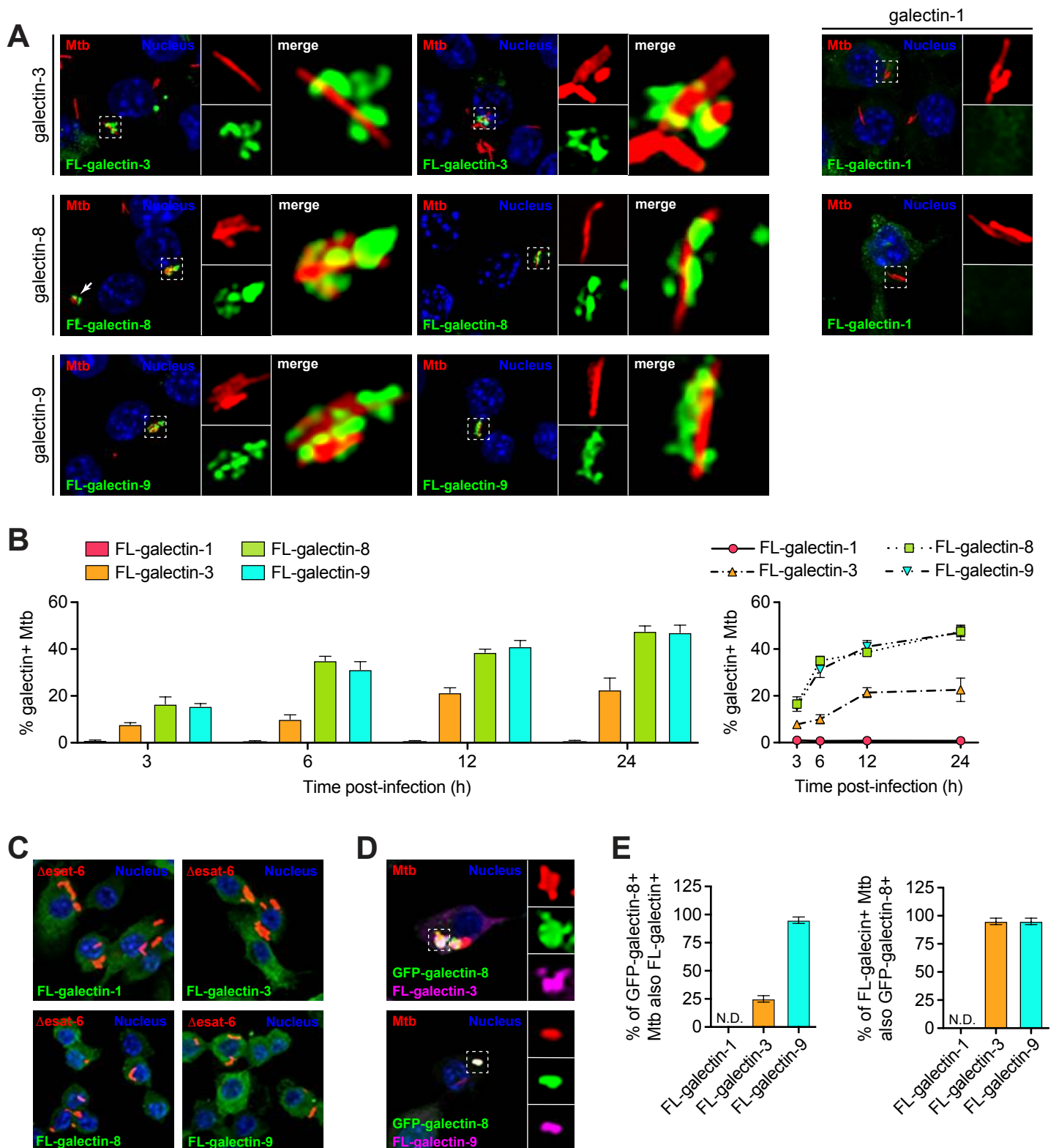
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## 134 **RESULTS**

### 135 **Galectins-3, -8, and -9 access the lumen of damaged Mtb-containing phagosomes to detect and** 136 **target cytosolically exposed bacilli**

137 Because galectins have previously been implicated in sensing phagosomal damage (Feeley et  
138 al., 2017; Thurston et al., 2012), we hypothesized that they may play a role in sensing Mtb in  
139 macrophages. To test if galectins were recruited to Mtb phagosomes early after infection, we generated  
140 3xFLAG-tagged expression constructs of four different galectins: galectins-1, -3, -8, and -9 (Fig. S1A-B).  
141 Galectins-3, -8, and -9 were chosen based on their post-translational modifications during Mtb infection  
142 (Budzik et al., 2020; Penn et al., 2018) and because previous studies in non-immune cells have found  
143 these galectins colocalized with intracellular pathogens (Thurston et al., 2012). Galectin-1 was chosen  
144 as a negative control. We stably expressed epitope-tagged galectins in RAW 264.7 cells, a murine  
145 macrophage-like cell line that are a common *ex vivo* infection model for Mtb since they are genetically  
146 tractable and respond robustly to Mtb infection (Hoffpauir et al., 2020; Watson et al., 2015). Using these  
147 cell lines, we infected with mCherry-expressing Mtb (Erdman strain), and at various times post-infection  
148 (3, 6, 12, 24 h), fixed coverslips and used immunofluorescence microscopy to assess 3xFLAG-galectin  
149 localization relative to intracellular Mtb (Fig. 1A-B). Galectins-8 and -9, and to a lesser extent galectin-3,  
150 were recruited to a sizeable population of Mtb, while galectin-1 was not. Colocalization was detectable at  
151 3 h post-infection and reached a maximum of ~45% galectin-8- or galectin-9-positive bacilli after 24 h.  
152 Galectin-3 was recruited to Mtb with similar dynamics, but was only recruited to a maximum of ~20% of

## Figure 1



**Figure 1. Galectins are recruited to Mtb-containing phagosomes. (A)** Immunofluorescence of RAW 264.7 cells stably expressing 3xFLAG (FL)-tagged galectins infected with wild-type (WT) mCherry-expressing Mtb (MOI = 1) 6 hr post-infection. Green, FL-galectin; red, mCherry Mtb; blue, DAPI. **(B)** Quantification of FL-galectin-positive Mtb (of indicated genotype) as shown in (A) at indicated time-points. **(C)** As in (A) but with cells infected with  $\Delta$ esat-6 mCherry-expressing Mtb. **(D)** Immunofluorescence of RAW 264.7 cells stably co-expressing GFP-galectin-8 and FL-galectin-3 or -9 infected with WT mCherry-expressing Mtb (MOI = 1) 6 hr post-infection. Green, GFP-galectin-8; magenta, FL-galectin; red, mCherry Mtb; blue, DAPI. **(E)** Quantification of GFP-galectin-8-positive and FL-galectin-3 or -9-positive Mtb shown in (D). GFP-galectin-8-positive Mtb that are also FL-galectin-3 or -9-positive (left) and FL-galectin-3 or -9-positive Mtb that are also GFP-galectin-8-positive (right). Error bars indicate S.D. of three coverslips per cell line in which at least 100 bacteria were assessed.



153 bacilli after 24 h. Galectin-1 did not colocalize with Mtb at any time point examined, making it a useful  
154 negative control for future experiments.

155 Next, we tested if the ESX-1 secretion system, and therefore phagosome permeabilization, was  
156 required for galectin recruitment. To do this, we infected 3xFLAG-galectin cells with mCherry-expressing  
157  $\Delta$ esat-6 Mtb (missing a key component for forming pores in the phagosomal membrane (Jonge et al.,  
158 2007)). Using immunofluorescence microscopy at 6 h post-infection, we did not observe colocalization of  
159 any galectin with  $\Delta$ esat-6 Mtb (Fig. 1C), which indicates that phagosomal permeabilization is required for  
160 galectin recruitment. Here and in future experiments, we examined the 6 h post-infection time point since  
161 this was the earliest that we observed peak galectin recruitment to Mtb (Fig. 1B). Together, these findings  
162 show that ESX-1-induced phagosomal damage is extensive enough to allow cytosolic proteins to access  
163 the lumen of the Mtb-containing phagosome.

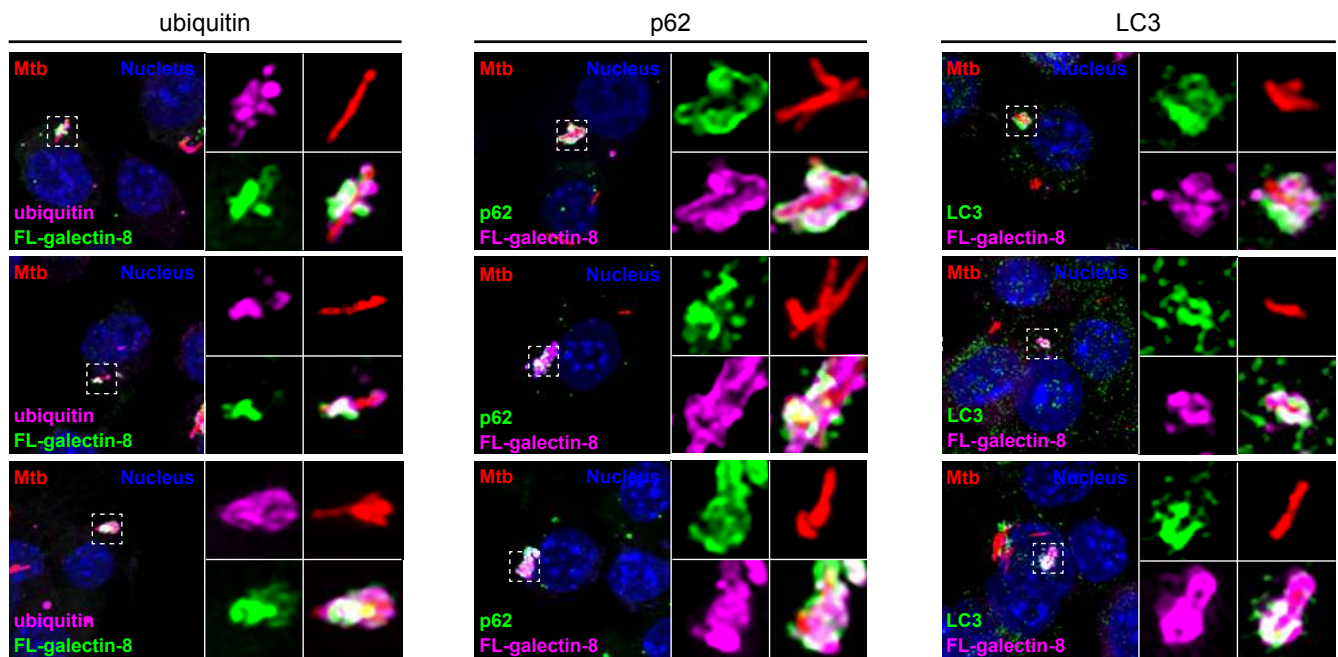
164 We next tested whether galectins-3, -8, and -9 were all recruited to the same Mtb-containing  
165 phagosomes. To do this, we stably co-expressed GFP-galectin-8 and 3xFLAG-galectin-3 or -9 in RAW  
166 264.7 cells and again infected them with mCherry Mtb. We found that galectin-8 and -9 colocalized in  
167 almost all instances (Fig. 1D-E). Likewise, galectin-3 was present on almost all galectin-8+ Mtb, but a  
168 large portion of galectin-8+ Mtb did not have galectin-3 present (Fig. 1D-E). This suggests that the same  
169 ~30% population of intracellular Mtb accumulates galectins-8 and -9, and sometimes galectin-3.

170 Based on previous reports and the size of the galectin+ Mtb population, we hypothesized that  
171 galectin+ Mtb-containing phagosomes would be positive for selective autophagy markers. To test this,  
172 we co-stained for 3xFLAG-galectin-8 and a panel of selective autophagy markers, including ubiquitin (the  
173 “eat me” signal), p62 (a selective autophagy adapter), and LC3 (the autophagosome marker). As  
174 predicted, the galectin-8+ Mtb were also positive for ubiquitin, p62, and LC3 at 6 h post-infection (Fig. 2).  
175 This indicates that galectin+ Mtb are indeed the same population of Mtb that are targeted to selective  
176 autophagy.

177

178 **Loss of galectin-8 decreases targeting of Mtb to selective autophagy**

## Figure 2



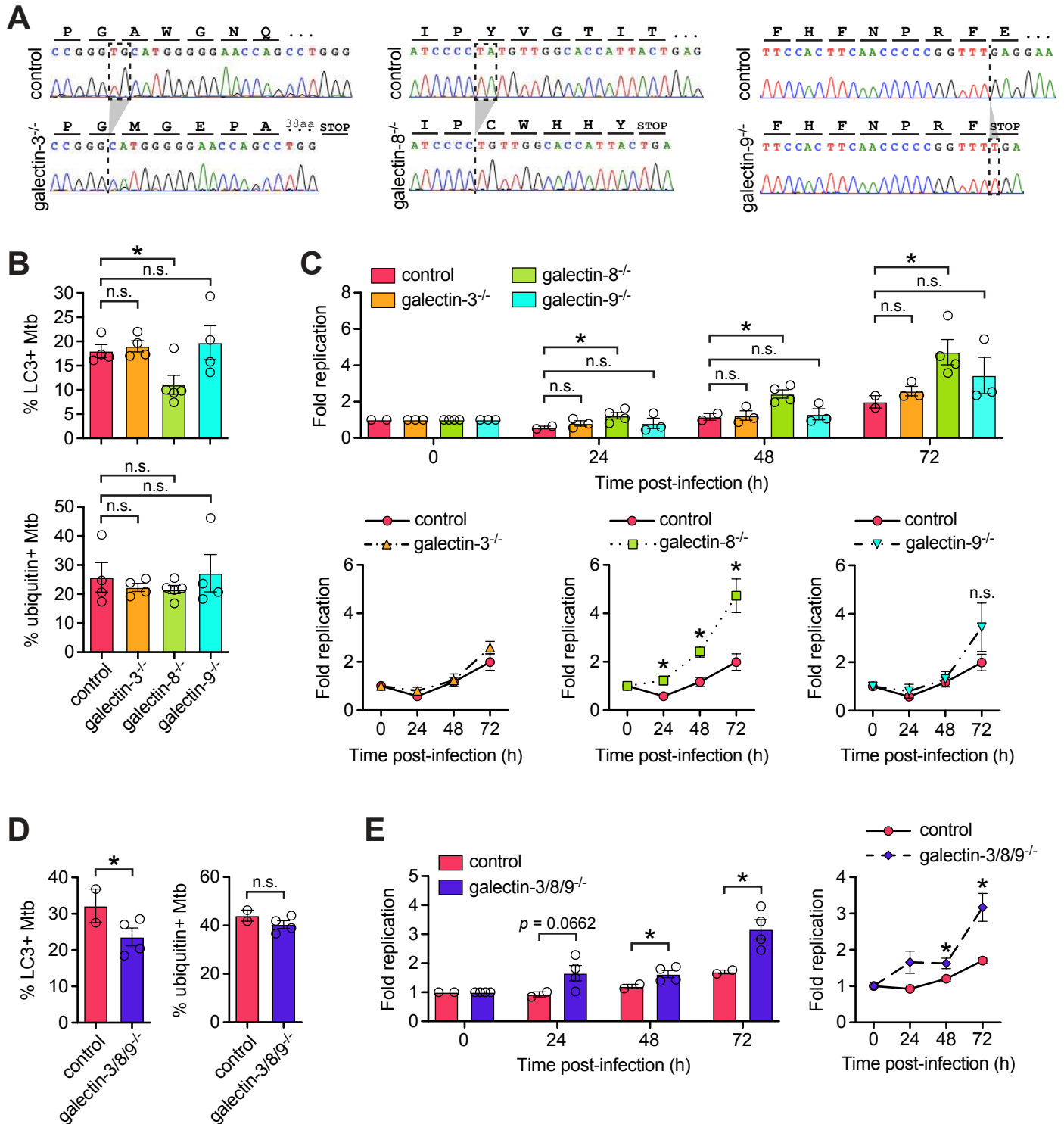
**Figure 2. Galectin-decorated Mtb-containing phagosomes colocalize with selective autophagy markers.** Immunofluorescence of RAW 264.7 cells stably expressing 3xFLAG (FL)-tagged galectin-8 infected with WT mCherry Mtb (MOI = 1) 6 hr post-infection co-stained for indicated selective autophagy marker (ubiquitin, p62, and LC3). Green and magenta, endogenous selective autophagy marker or FL-galectin-8 (as indicated); red, mCherry Mtb; blue, DAPI.

179 We next sought to determine if the recruitment of galectins is required for targeting Mtb to  
180 antibacterial selective autophagy. To do this, we used a lentiviral CRISPR/Cas9 system to mutate the  
181 genes encoding galectins-3, -8, or -9 (*Lgals3*, *Lgals8*, *Lgals9*) in RAW 264.7 cells. We designed small  
182 guide RNAs (sgRNAs) targeting the first one to two coding exons of each galectin gene; we used GFP-  
183 targeted sgRNAs as negative controls. After transducing RAW 264.7 cells stably expressing FLAG-Cas9  
184 with lentiviral sgRNAs constructs, we antibiotic-selected cells, isolated clonal populations, and validated  
185 homozygous mutation by sequencing the targeted region. We chose clonal populations that had one or  
186 two basepair insertions or deletions that resulted in frameshift mutations early in the transcript (exon 1 or  
187 2) (Fig. 3A). To limit the possibility of off-target and bottleneck effects, we used at least three clonal  
188 populations for each gene, and these were derived from two different gRNAs per gene. Since we were  
189 unable to identify commercial antibodies that reliably detected the three mouse galectins, we further  
190 validated loss of gene expression in the knockout cell lines using RT-qPCR since the mutated transcripts  
191 should be degraded via nonsense mediated decay. As expected, all of the knockout cell lines had  
192 significantly diminished mRNA expression of the sgRNA-targeted galectin (Fig. S1C).

193 Next, we tested if these knockout cell lines could efficiently target Mtb to selective autophagy. We  
194 infected with mCherry Mtb, stained for the autophagy marker LC3, and quantified the percentage of  
195 targeted bacteria. Compared to control cell lines (GFP sgRNAs), galectin-8<sup>-/-</sup> cell lines had less  
196 (approximately 50% less) LC3<sup>+</sup> bacteria at 6 h post-infection (Fig. 3B, top). This was specific to galectin-  
197 8 as galectin-3<sup>-/-</sup> and galectin-9<sup>-/-</sup> cell lines had similar percentages of LC3<sup>+</sup> Mtb compared to controls.  
198 These cell lines all had similar proportions of ubiquitin<sup>+</sup> Mtb (Fig. 3B, bottom), which suggests that  
199 galectin recruitment is independent of ubiquitination.

200 To test how this defect in targeting impacts Mtb survival/replication in macrophages, we measured  
201 bacterial replication using an Mtb strain constitutively expressing luxBCADE. With this strain, at various  
202 timepoints post-infection, we could use luminescence as a proxy to monitor Mtb replication in numerous  
203 cell lines (Budzik et al., 2020; Hoffpaur et al., 2020; Penn et al., 2018). In control cells, Mtb replication is  
204 well-controlled; after 24 h bacterial burdens decrease before Mtb begins to slowly replicate intracellularly

## Figure 3



**Figure 3. Galectin-8 is required to efficiently target Mtb to selective autophagy to control Mtb replication in macrophages. (A)** Representative chromatograms from galectin-3, -8, and -9 knockout cell lines indicating the nature of nonsense mutations introduced via CRISPR/Cas9. **(B)** Quantification of LC3-positive (top) and ubiquitin-positive (bottom) Mtb in control (sgRNAs targeting GFP) or individual galectin knockout RAW 264.7 cell lines at 6 h post-infection. Circles represent data for each clonally selected cell line (at least two cell lines per sgRNA and two sgRNAs per galectin gene). **(C)** Fold replication of luxBCADE Mtb (MOI = 1) in control and galectin knockout cell lines at indicated time points. Data normalized to t=0 h. **(D-E)** As in (B-C) but with RAW 264.7 cell lines in which all three galectins are knocked out. Error bars indicate S.E.M. of knockout cell lines, for IF, at least 300 bacteria per cell line were assessed. \*,  $p < 0.05$ ; n.s., not significant.

205 at later time points (Fig. 3C). However, in galectin-8<sup>-/-</sup> macrophages, but not galectin-3<sup>-/-</sup> or galectin-9<sup>-/-</sup>  
206 macrophages, Mtb was not controlled at 24 h post-infection and instead replicated ~2-fold (Fig. 3C). In  
207 addition, the higher bacterial burdens in galectin-8<sup>-/-</sup> cells persisted over 72 h of infection. This indicates  
208 that the defective selective autophagy targeting in galectin-8<sup>-/-</sup> macrophages results in diminished control  
209 of Mtb survival/replication, and together, these data suggest that galectin-8 in particular is required for  
210 targeting Mtb to antibacterial selective autophagy.

211 Because several galectins are recruited to Mtb during infection, we next investigated whether they  
212 served redundant functions in targeting Mtb to selective autophagy. We used a lentiviral sgRNA array  
213 construct to simultaneously express the most efficient galectin-specific sgRNAs or as a negative control,  
214 GFP sgRNAs, in FLAG-Cas9-expressing RAW 264.7 cells. As with the single knockout lines, we isolated  
215 clonal cell populations, confirmed homozygous mutation of all three galectin genes, and validated the  
216 triple knockout cell lines by measuring galectin transcript levels (Fig. S1D). We infected the galectin-3/8/9<sup>-/-</sup>  
217 <sup>-/-</sup> triple knockout cells and GFP sgRNA control cells with mCherry Mtb and used immunofluorescence  
218 microscopy to quantify selective autophagy targeting. Compared to controls, the galectin-3/8/9<sup>-/-</sup> cell lines  
219 had fewer LC3<sup>+</sup> Mtb 6 h post-infection, but similar numbers of ubiquitin<sup>+</sup> Mtb (Fig. 3D). When infected  
220 with luxBCADE Mtb, the galectin-3/8/9<sup>-/-</sup> cell lines also had higher Mtb survival/replication compared to  
221 controls (Fig. 3E-F). Surprisingly, the magnitude of the defect in the galectin-3/8/9<sup>-/-</sup> triple knockout cells  
222 (~2-fold defect) phenocopied that of the galectin-8<sup>-/-</sup> single knockout lines (~2- to 2.5-fold defect),  
223 suggesting that these three galectins do not serve redundant functions, and instead galectin-8 has a  
224 privileged role in targeting Mtb to selective autophagy.

225

### 226 **Galectin-8 interacts with diverse proteins involved in exosome secretion, membrane trafficking,** 227 **and selective autophagy**

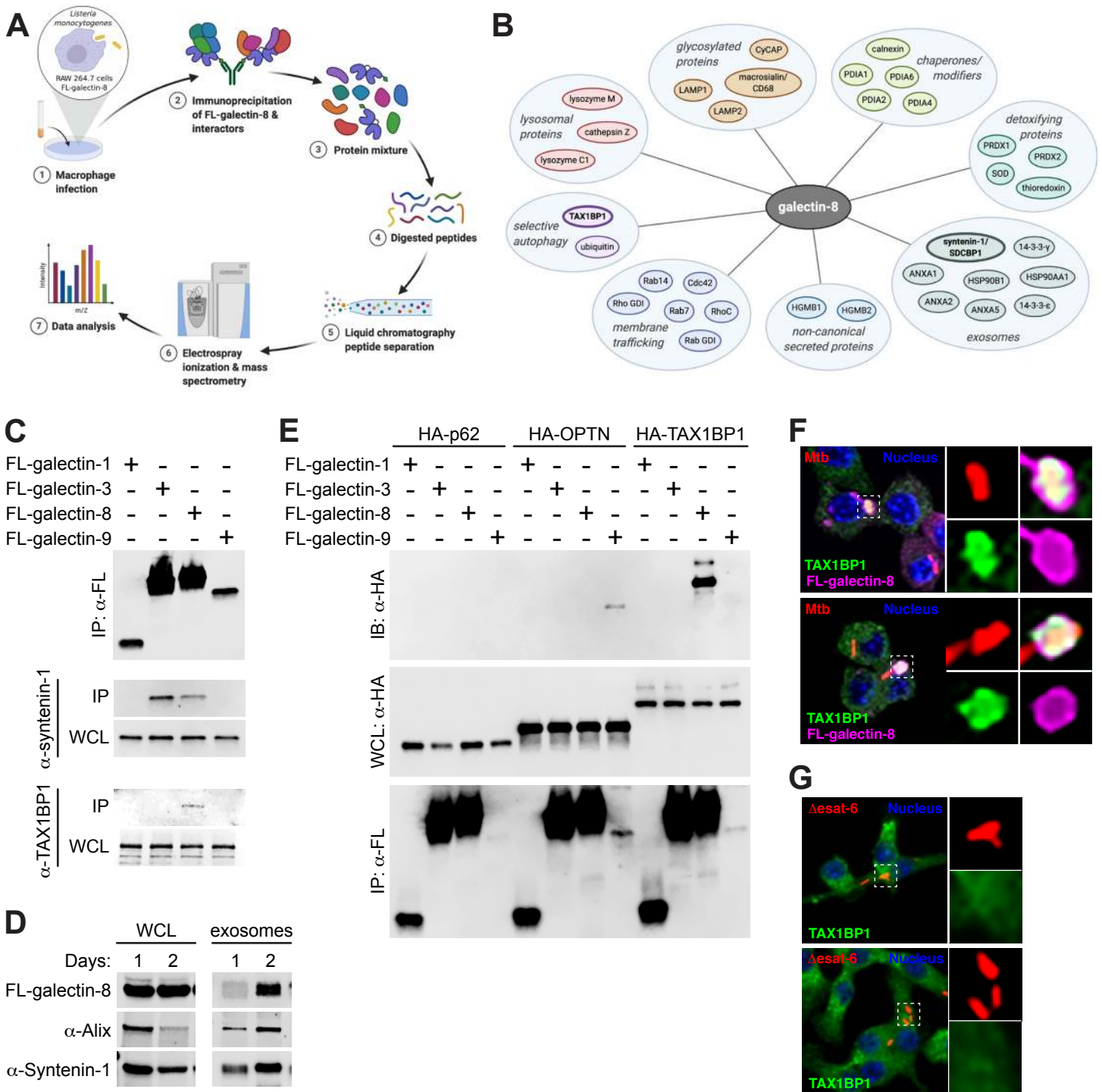
228 To gain a deeper understanding of how galectin-8 promotes targeting of Mtb to selective  
229 autophagy, we used an unbiased mass-spec approach. We predicted that galectin-8 may have one or  
230 more specific binding partners that would help explain why loss of galectin-8 in particular decreased LC3

231 recruitment to the Mtb-containing phagosome. Due to technical limitations resulting from Mtb's  
232 classification as a Biosafety Level 3 (BSL3) pathogen, we turned to *Listeria monocytogenes*, a BSL2  
233 pathogen that also elicits a type I IFN response, can be targeted to selective autophagy, and recruits  
234 galectins-3, -8, and -9 (Manzanillo et al., 2012; Mitchell et al., 2015; Thurston et al., 2012). To increase  
235 the population of *L. monocytogenes* targeted to selective autophagy, we used a strain lacking ActA, a  
236 protein that enables mobility within the host cell and therefore helps bacteria evade autophagy (Mitchell  
237 et al., 2015). We infected RAW 264.7 cells stably expressing 3xFLAG-galectin-8 with  $\Delta$ actA *L.*  
238 *monocytogenes* at a multiplicity of infection (MOI) of 5, and immunoprecipitated 3xFLAG-galectin-8.  
239 Proteins associated with galectin-8 were identified using LC/MS (Fig. 4A).

240 The protein interacting partners identified by IP-LC/MS provided insight into several novel aspects  
241 of galectin-8 biology. First, consistent with galectin-8 recognizing damaged phagosomes, endosomes,  
242 and lysosomes, we found lysosomal proteins (cathepsin Z, lysozyme M, lysozyme C1), highly  
243 glycosylated proteins (LAMP1, LAMP2, macrosialin/CD68, cyclophilin C-associated protein),  
244 chaperones/modifiers of glycosylated proteins (calnexin, protein disulfide isomerases [PDIA1, PDIA3,  
245 PDIA4, PDIA6]), and detoxifying enzymes (thioredoxin, superoxide dismutase, peroxiredoxins  
246 [PRDX1, PRDX2]). Additionally, we identified several galectin-8 binding partners with known roles in  
247 membrane trafficking (Rab7, Rab14, RhoC, Cdc42, Rab GDI [GDP dissociation inhibitor], Rho GDI) and  
248 cytoskeleton rearrangements (EFhd2, profilin, talin-1, gelsolin, F-actin capping proteins, macrophage  
249 capping protein), which are all consistent with galectin-8's role in recognizing damaged endosomes and  
250 lysosomes.

251 Interestingly, we identified several proteins that, like galectins, are secreted through a non-  
252 canonical pathway that does not require a signal sequence, including HGMB1 (Gardella et al., 2002; Li  
253 et al., 2020). Also identified were a panel of proteins associated with exosome secretion, a form of non-  
254 canonical secretion, including syntenin-1/SDCBP, HSP90AA1, HSP90B1, ANXA1, ANXA2, ANXA5, 14-  
255 3-3-epsilon/YWAHAE, and 14-3-3-gamma/YWAHAG (Baietti et al., 2012; Gonzalez-Begne et al., 2009;  
256 Guha et al., 2019; Lauwers et al., 2018). Using co-immunoprecipitations of 3xFLAG-tagged galectins

## Figure 4



**Figure 4. Galectin-8 interacts with exosome-associated proteins and selective autophagy adapter TAX1BP1.** (A) Schematic of approach for immunoprecipitation and mass spectrometry (IP-LC/MS) identification of galectin-8 binding partners in macrophages during intracellular bacterial infection. (B) Proteins identified by IP-LC/MS with galectin-8. (C) Co-immunoprecipitation (IP) of 3xFLAG (FL)-tagged galectins ectopically expressed in HEK293T cells. Whole cell lysates (WCL) and co-IPs probed for endogenous syntenin-1 and TAX1BP1. (D) WCL and exosomes from FL-galectin-8-expressing RAW 264.7 cells cultured for indicated number of days to assess exosome accumulation in cell culture media. (E) Directed co-IPs of FL-galectins and HA-tagged selective autophagy adapters. (F) Immunofluorescence of RAW 264.7 cells stably expressing FL-galectin-8 and co-stained for endogenous TAX1BP1 infected with WT mCherry-expressing Mtb (MOI = 1) 6 h post-infection. Green, endogenous TAX1BP1; magenta, FL-galectin-8; red, mCherry Mtb; blue, DAPI. (G) Immunofluorescence of RAW 264.7 cells infected with  $\Delta$ esat-6 mCherry Mtb and stained for endogenous TAX1BP1 at 6 h post-infection. Green, endogenous TAX1BP1; red,  $\Delta$ esat-6 Mtb; blue, DAPI. Panels (A-B) made with BioRender.com.

257 ectopically expressed in HEK293T cells, we confirmed this interaction between galectin-8 and  
258 endogenous syntenin-1 (Fig. 4C). Interestingly, this interaction was not unique to galectin-8 since  
259 galectin-3, but not galectins-1 or -9, also interacted with syntenin-1. These observations led us to  
260 hypothesize that galectin-8 could be secreted via exosomes. To test this, we isolated exosomes from the  
261 cell culture supernatant of RAW 264.7 cells and found that 3xFLAG-galectin-8, along with the exosomal  
262 proteins Alix and syntenin-1, were present in exosome preps (Fig. 4D). Moreover, the amount of  
263 exosomal galectin-8, Alix, and syntenin increased over time as exosomes accumulated in the cell culture  
264 media. Together, these data suggest that release in exosomes may be a key mechanism of secretion for  
265 extracellular galectins.

266 Finally, our mass spectrometry analysis identified ubiquitin, which is consistent with our  
267 observation that galectin-8 colocalizes with ubiquitin+ Mtb (Fig. 2), and it corroborates recent studies  
268 using global proteomics approaches that found galectin-8 itself is ubiquitinated during Mtb infection  
269 (Budzik et al., 2020; Penn et al., 2018). We also identified TAX1BP1 as a galectin-8 interacting protein.  
270 While TAXBP1 has been previously characterized as a selective autophagy adaptor with ubiquitin- and  
271 LC3-binding domains, it is not known to interact with galectins. We hypothesized that galectin-8 could  
272 augment selective autophagy of Mtb by binding to TAX1BP1 and promoting recruitment of downstream  
273 autophagy machinery.

274

### 275 **Galectin-8 interacts with TAX1BP1 independently of ubiquitination**

276 We first confirmed the galectin-8/TAX1BP1 interaction using HEK293T cells ectopically  
277 expressing 3xFLAG-galectins and found that endogenous TAX1BP1 immunoprecipitated specifically with  
278 galectin-8 (Fig. 4C). To further probe the specificity of the galectin-8/TAX1BP1 interaction, we generated  
279 HA-tagged expression constructs for several selective autophagy adaptors, including TAX1BP1, p62,  
280 and optineurin/OPTN. We then tested the interaction between each galectin and adaptor by co-  
281 expressing pairs in HEK293T cells and performing directed co-IPs. Remarkably, we found that galectin-  
282 8 specifically interacted with TAX1BP1 and no other adaptors, and HA-TAX1BP1 interacted specifically

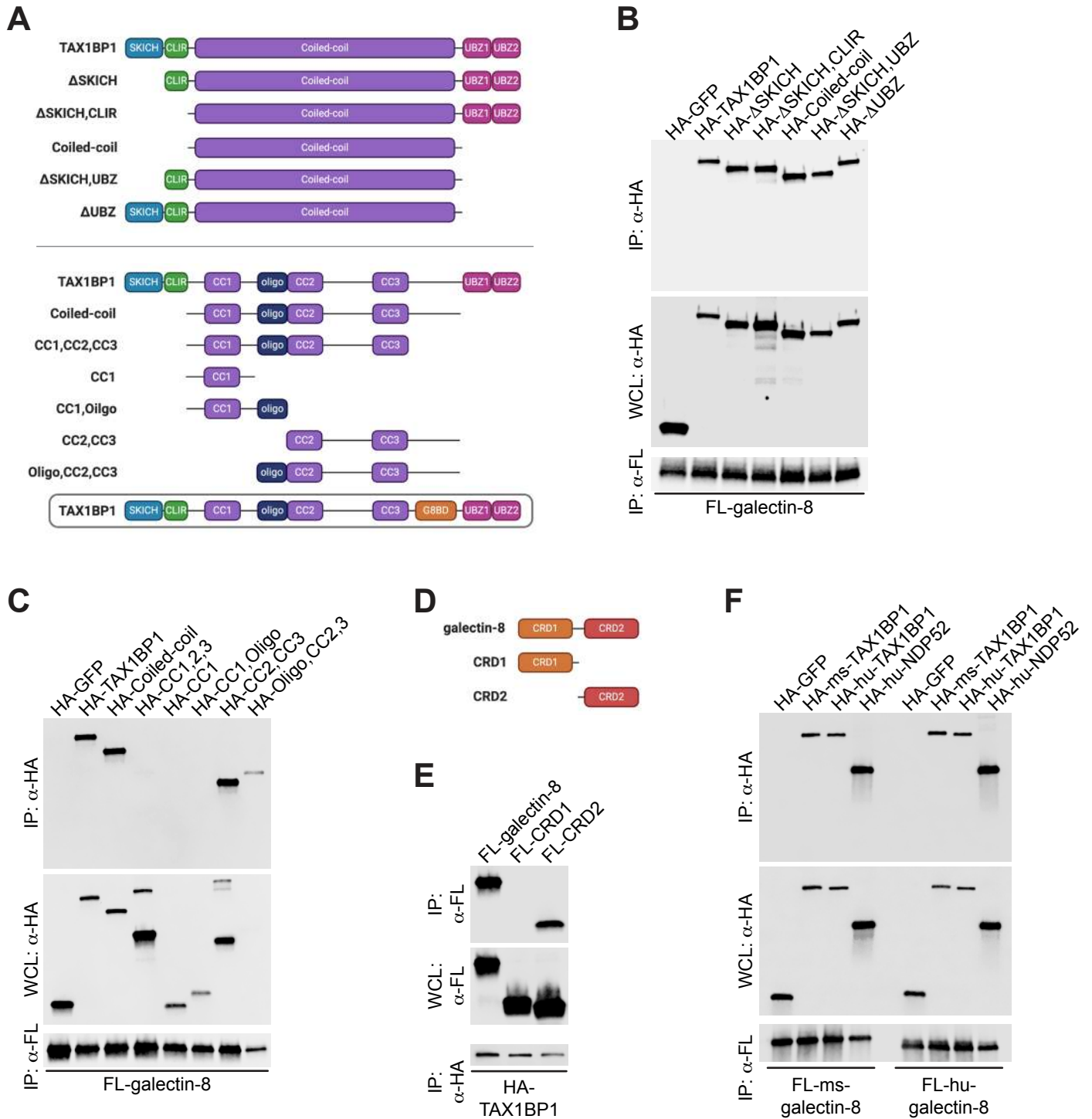


283 with only with galectin-8 and no other galectins (Fig. 4E). We also detected an unexpected but seemingly  
284 specific interaction between galectin-9 and OPTN (Fig. 4E). These highly specific protein-protein  
285 interactions are surprising since there is a high degree of similarity between galectins (Fig. S2 A-B).

286 We next examined the localization of TAX1BP1 during Mtb infection. We infected RAW 264.7  
287 cells expressing 3xFLAG-galectin-8 with mCherry Mtb and used immunofluorescence microscopy to  
288 visualize endogenous TAX1BP1. TAX1BP1 colocalized with galectin-8+ Mtb (Fig. 4F), and we found near  
289 complete overlap in the TAX1BP1+ and galectin-8+ populations. Furthermore, in cells infected with  
290  $\Delta$ esat-6 Mtb, TAX1BP1 did not colocalize with Mtb, indicating that like galectins (Fig. 1C) and other  
291 adapters (Watson et al., 2012), phagosomal damage and/or cytosolic exposure is required for the  
292 recruitment of TAX1BP1 (Fig. 4G). Because we detected an interaction between galectin-9 and OPTN,  
293 we performed similar experiments co-staining for OPTN. However, we found that endogenous OPTN did  
294 not colocalize with Mtb at any time points examined (Fig. S2C). However, when we stably expressed  
295 3xFLAG-OPTN in RAW 264.7 cells, we observed low levels of colocalization (Fig. S2C), suggesting that  
296 while OPTN is capable of being recruited to the Mtb-containing phagosome, it is unlikely to play a  
297 substantial role in the early targeting of Mtb to selective autophagy under normal conditions. Because  
298 several galectins (galectins-3, -8, and -9) and selective autophagy adapters (TAX1BP1, p62) are all  
299 recruited to the same population of Mtb-containing phagosomes, the highly specific galectin-8/TAX1BP1  
300 interaction is particularly noteworthy.

301 To investigate the mechanisms by which these proteins interact, we made a series of truncations  
302 of both galectin-8 and TAX1BP1. TAX1BP1 contains several annotated domains, including a SKICH  
303 domain, an LC3-interacting region (LIR), a large coiled-coil domain, and two ubiquitin-binding zinc fingers  
304 (UBZs)(Fig. 5A). Because galectin-8 itself is likely ubiquitinated during infection, we predicted that  
305 TAX1BP1 binds galectin-8 via its UBZ domains. Surprisingly, when we performed directed co-IPs  
306 between galectin-8 and a panel of TAX1BP1 truncations, we found that the UBZ domains of TAX1BP1  
307 were dispensable for its interaction with galectin-8 in this system (Fig. 5B). Instead, only the coiled-coil  
308 domain was required for interaction. To further narrow the region required for interaction with galectin-8,

## Figure 5



309 we tested additional truncations of TAX1BP1 that included combinations of the N- and C-terminals of the  
310 coiled-coil domain, an annotated oligomerization domain, and three smaller coiled-coil domains (Fig. 5A).  
311 In co-IPs with galectin-8 and these additional TAX1BP1 truncations, we found that the C-terminal portion  
312 of the coiled-coil domain was required and sufficient for this interaction (Fig. 5C). We propose calling this  
313 region of TAX1BP1 the galectin-8-binding domain (G8BD)(Fig. 5A). We next investigated truncations of  
314 galectin-8, which contains two carbohydrate recognition domains (CRDs) that are connected by a short  
315 flexible linker (Fig. 5D). In directed IPs, we found that the C-terminal CRD domain (CRD2), but not the  
316 N-terminal CRD (CRD1), interacted with TAX1BP1 (Fig. 4E). Together, these biochemical experiments  
317 indicate that TAX1BP1 has evolved a ubiquitin-independent mechanism to specifically interact with  
318 galectin-8.

319 A previous study found that in non-immune cells, galectin-8 interacts with another selective  
320 autophagy adaptor, NDP52, which has a domain structure highly similar to TAX1BP1 (Fig. S2D)(Thurston  
321 et al., 2012). This study found that, similar to our findings in TAX1BP1, human NDP52 interacts with  
322 galectin-8 via the C-terminal region of NDP52's comparatively smaller coiled-coil domain. Because of  
323 these similarities, we wanted to test the conservation of the TAX1BP1/galectin-8 interaction. To do this,  
324 we co-expressed human 3xFLAG-galectin-8 with human HA-TAX1BP1 or human HA-NDP52 and  
325 performed co-IPs. Consistent with previous reports, galectin-8 interacted with NDP52 (Fig. 5F).  
326 Importantly, human galectin-8 also interacted with human TAX1BP1 (Fig. 5F). This previously  
327 unidentified interaction indicates that galectin-8 can interact with both NDP52 and TAX1BP1 in humans.  
328 Based on our previous studies, the mouse gene encoding NDP52 appears to be disrupted by repetitive  
329 elements and lacks the regions previously shown to interact with galectin-8. Therefore, while the reported  
330 interaction between NDP52 and galectin-8 is likely not at play in mouse cells, it appears that human cells  
331 have evolved galectin-8 binding partners that may serve redundant functions. Finally, mouse galectin-8  
332 can interact with human TAX1BP1 and human NDP52, and human galectin-8 can interact with mouse  
333 TAX1BP1 (Fig. 5F), which suggests that galectin-8, TAX1BP1, and their biochemical interactions are  
334 highly conserved.

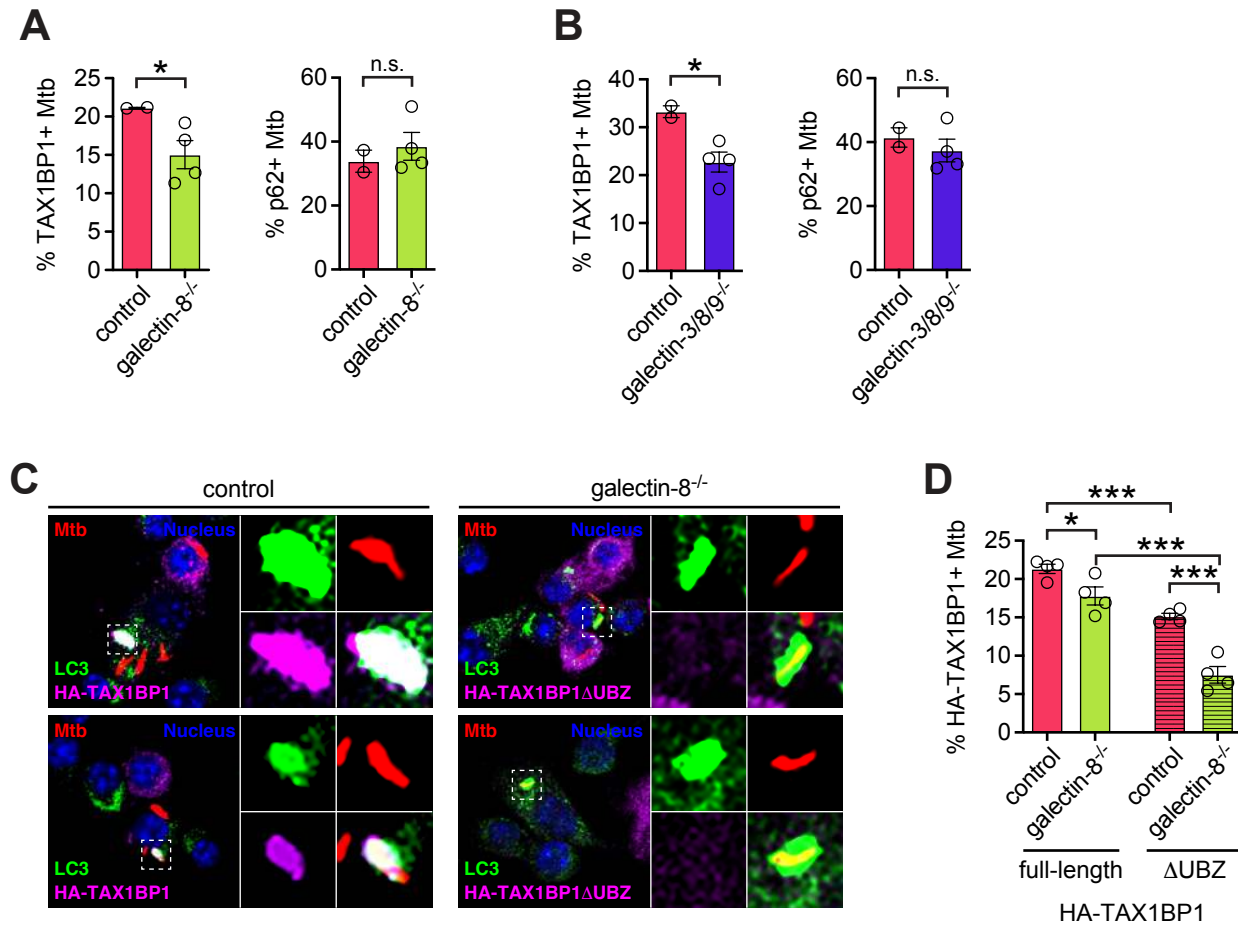
335

336 **TAX1BP1 can be recruited to Mtb-containing phagosomes by binding galectin-8 or ubiquitinated**  
337 **proteins**

338 To assess how the galectin-8/TAX1BP1 interaction influences targeting of Mtb to selective  
339 autophagy, we looked at the recruitment of TAX1BP1 to Mtb in galectin-8<sup>-/-</sup> cells. The percentage of  
340 TAX1BP1+ Mtb in both galectin-8<sup>-/-</sup> and galectin-3/8/9<sup>-/-</sup> cell lines was lower compared to controls (Fig.  
341 6A-B). This defect in recruitment was specific to TAX1BP1, though, since the number of p62+ Mtb was  
342 similar in knockout and control cells (Fig. 6A-B).

343 Because a sizeable population of Mtb were TAX1BP1+ even in the absence of galectin-8, we next  
344 investigated how specific domains of TAX1BP1 might contribute to its colocalization with Mtb. We  
345 predicted that because TAX1BP1 has UBZ domains, perhaps it could be recruited to Mtb in the absence  
346 of galectin-8 by binding to other ubiquitinated substrates surrounding the Mtb-containing phagosome. To  
347 test this, we stably expressed full-length HA-TAX1BP1 or HA-TAX1BP1 lacking the UBZ domains (HA-  
348 TAX1BP1 $\Delta$ UBZ) in control and galectin-8<sup>-/-</sup> cell lines. Then, at 6 h post-infection with mCherry Mtb, we  
349 performed immunofluorescence microscopy to quantify the number of HA-TAX1BP1+ bacteria (Fig. 6C).  
350 Consistent with experiments in Fig. 6A, which examined endogenous TAX1BP1, full-length HA-TAX1BP1  
351 was recruited less efficiently in galectin-8<sup>-/-</sup> cells (Fig. 6D), again indicating the galectin-8/TAX1BP1  
352 interaction is required for TAX1BP1 recruitment. Furthermore, in control cells expressing HA-  
353 TAX1BP1 $\Delta$ UBZ, even fewer Mtb were TAX1BP1+ (Fig. 6D), suggesting that TAX1BP1's ability to bind  
354 ubiquitinated substrates is also required for its recruitment to Mtb. Finally, in support of our prediction,  
355 HA-TAX1BP1 $\Delta$ UBZ was recruited least efficiently in galectin-8<sup>-/-</sup> cells (Fig. 6D), suggesting that both  
356 binding capabilities are involved in recruiting TAX1BP1 to Mtb. The residual recruitment of TAX1BP1 to  
357 Mtb in the absence of both galectin-8 and UBZ domains could be mediated by TAX1BP1's LIR (LC3  
358 interacting region) or other interactions or oligomerization with endogenous wild-type TAX1BP1.  
359 Together, these data demonstrate that TAX1BP1 can be recruited to damaged Mtb-containing

## Figure 6



**Figure 6. TAX1BP1 is recruited to Mtb-containing phagosomes by both its UBZ domain and its interaction with galectin-8.** (A) Quantification of TAX1BP1- or p62-positive Mtb in control galectin-8 knockout RAW 264.7 cell lines at 6 h post-infection. Circles represent data for individual clonally selected cell lines. (B) As in (A) but with RAW 264.7 cell lines in which all three galectins are knocked out. (C) Immunofluorescence of control or galectin-8 knockout RAW 264.7 cells stably expressing full-length HA-TAX1BP1 or truncated HA-TAX1BP1ΔUBZ that is missing its ubiquitin-binding domain. Cells were infected with WT mCherry-expressing Mtb (MOI = 1) and harvested at 6 h post-infection. Green, LC3; magenta, HA-TAX1BP1 variants; red, mCherry Mtb; blue, DAPI. (D) Quantification of indicated variant HA-TAX1BP1-positive Mtb in indicated genotype. Error bars indicate S.E.M. of knockout cell lines in which at least 300 bacteria per cell line were assessed. \*,  $p < 0.05$ ; \*\*\*,  $p < 0.005$ ; n.s., not significant.

360 phagosome by at least two independent mechanisms: binding to galectin-8 via its coiled-coil domain and  
361 binding to ubiquitinated substrates.

362

### 363 **Overexpression of galectins augments targeting to selective autophagy**

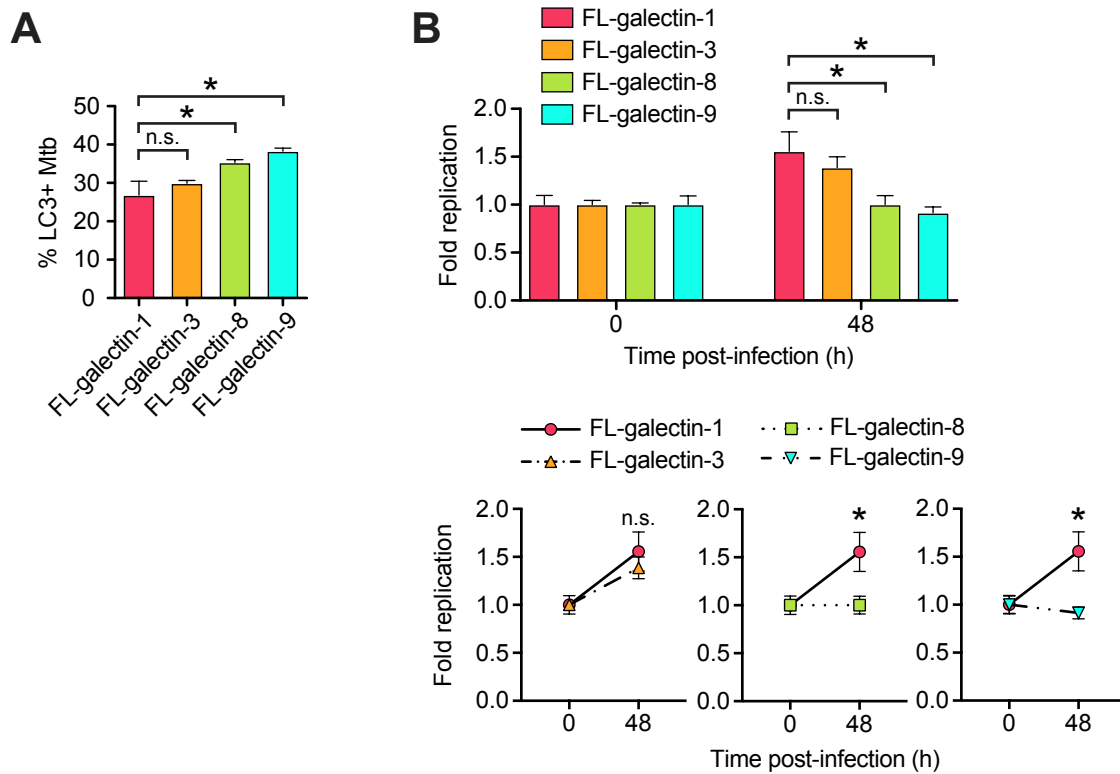
364 Finally, having characterized the requirement of galectins for targeting Mtb to selective autophagy,  
365 we next tested how overexpression of galectins might impact this pathway. RAW 264.7 cells  
366 overexpressing 3xFLAG-galectin-8 or galectin-9 had a small but significant increase in LC3+ Mtb at 6 h  
367 post-infection compared to cells overexpressing FL-galectin-1 (Fig. 7A). Importantly, the increased  
368 targeting in FL-galectin-8 and -9 cells translated to a significant increase in macrophages' ability to control  
369 Mtb replication as measured by luxBCADE Mtb (Fig. 7B). Overexpression of FL-galectin-3 had a  
370 moderate effect on selective autophagy targeting and controlling Mtb replication/survival, which is  
371 consistent with its intermediate recruitment phenotype (Fig. 1A-B). Together, these data indicate that  
372 overexpression of galectins substantially enhances macrophages' ability to recognize and respond to  
373 Mtb infection.

374

### 375 **DISCUSSION**

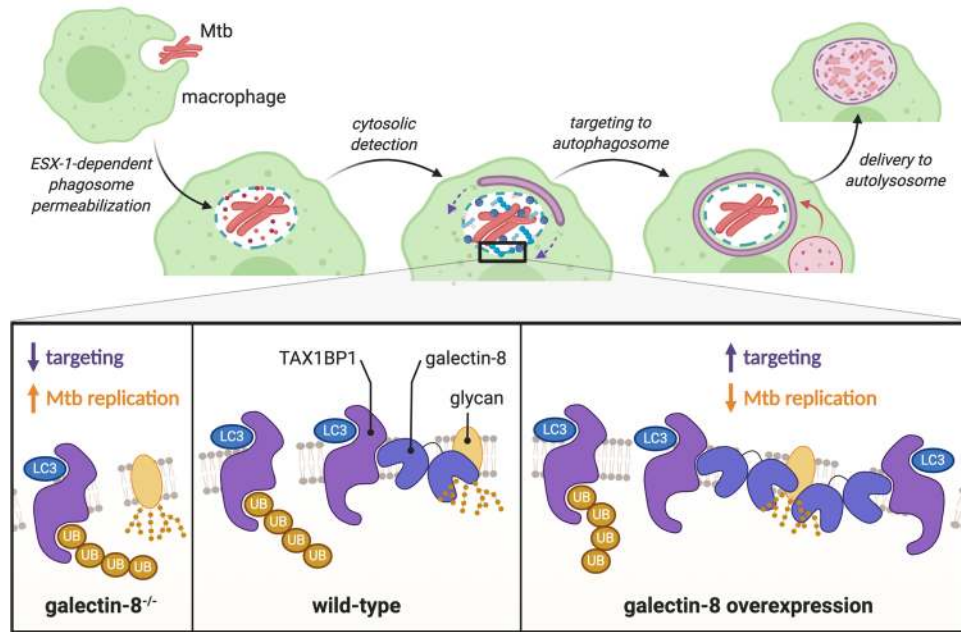
376 Selective autophagy is a critical pathway employed by macrophages to control Mtb infection. Here  
377 we characterized the involvement of galectins, a family of damage/danger sensors, in the selective  
378 autophagy response to Mtb (Fig. 8). Of the galectins we studied, we found that galectin-8, but not galectin-  
379 3 or -9, was required for controlling Mtb infection in macrophages. This is somewhat surprising since all  
380 three galectins were recruited to the phagosome. However, the specific requirement of galectin-8 may  
381 be due to its interaction with the selective autophagy adapter TAX1BP1, which a recent report found to  
382 be required for targeting Mtb to selective autophagy and controlling Mtb replication in macrophages  
383 (Budzik et al., 2020). Our data indicate that TAX1BP1 can be recruited to the Mtb-containing phagosome  
384 in two ways: by binding directly to galectin-8, which is recruited directly to damaged Mtb-containing  
385 phagosomes, and by binding to ubiquitinated substrates. Consistently, this two-pronged recruitment of

## Figure 7



**Figure 7. Overexpression of galectin-8 increases targeting and controls replication of Mtb.** (A) Quantification of LC3-, TAX1BP1-, p62-, and ubiquitin-positive Mtb in RAW 264.7 cells overexpressing 3xFLAG (FL)-tagged galectins at 6 h post-infection. (B) Fold replication of luxBCADE Mtb (MOI = 1) in FL-galectin overexpression cell lines at indicated time points. Data normalized to t=0 h. Error bars indicate S.D. of overexpression cell lines; for IF, at least 300 bacteria per cell line were assessed. \*,  $p < 0.05$ ; n.s., not significant.

## Figure 8



**Figure 8. Galectin-8 and TAX1BP1 recognize and target Mtb to selective autophagy in macrophages.** Schematic representation of how Mtb is detected by macrophages. Galectin-8 binds to cytosolically exposed glycans in the lumen of damaged Mtb-containing phagosomes. TAX1BP1 is recruited to these damaged phagosomes via its interaction with galectin-8 as well as through its interaction with ubiquitinated substrates. Deletion of galectin-8 results in less targeting of Mtb and increased survival/replication, while overexpression of galectin-8 leads to increased targeting and less Mtb replication. Made with BioRender.com.



386 an adaptor, via galectin-8 and via ubiquitinated substrates, has been observed for NDP52 in HeLa cells  
387 infected with *S. Typhimurium* (Thurston et al., 2012). Since NDP52 and TAX1BP1 are highly related  
388 selective autophagy adapter it is perhaps not surprising that they have similar functional profiles. The  
389 curious similarities and apparent redundancies between adapters emphasize the importance of  
390 understanding the nature of their specific biological functions. Many important questions remain to be  
391 explored, including whether TAX1BP1 and NDP52 serve truly redundant roles in human autophagy or if  
392 one has evolved a particularly important function in other cell types.

393 Our experiments demonstrate that even early during infection, when Mtb appears to be enclosed  
394 inside a vacuole, there is sufficient disruption of the phagosomal membrane to permit entry of host factors  
395 into the lumen of the Mtb-containing phagosome. As a result, there is likely substantial exposure of both  
396 pathogen-associated and damage-associated molecular patterns (PAMPs and DAMPs) very early during  
397 Mtb infection. Some of the host pattern recognition receptors that detect these danger signals are known,  
398 including cGAS and now galectins, and these studies indicate that the molecular environment around the  
399 Mtb-containing phagosomes is extremely complex and is crowded with many proteins involved in various  
400 host response pathways: cGAS (STING/TBK1/TRIM14/IRF3), galectins, and ubiquitin (adapters [p62,  
401 TAX1BP1, NDP52, NBR1], LC3s/GABARAPs, E3 ubiquitin ligases [Parkin, TRIM16, Smurf]). However,  
402 the molecular mechanistic links between these different proteins and pathways remain somewhat  
403 obscure. As a kinase, TBK1 can phosphorylate adapters like OPTN, NDP52, and p62 (Richter et al.,  
404 2016; Wild et al., 2011), and phosphorylation of OPTN by TBK1 can increase its affinity for ubiquitin.  
405 However, it remains unknown whether TBK1 activation influences adapters' affinity for ubiquitin, LC3, or  
406 galectins during Mtb infection. Furthermore, the E3 ubiquitin ligases Parkin, Smurf1, and TRIM16  
407 colocalize with Mtb and contribute to the ubiquitin cloud surrounding bacteria, but how these E3 ligases  
408 are activated upon Mtb infection and what proteins each E3 modifies are lingering unanswered questions.  
409 Finally, since Mtb is an exquisitely evolved pathogen, it is very likely that yet-to-be-identified bacterial  
410 proteins are intimately involved in these processes. Indeed, the recent discovery of a ubiquitin-binding

411 protein (Rv1468c) on Mtb's surface suggests that Mtb does have mechanisms for modulating the host's  
412 selective autophagy pathway (Chai et al., 2019).

413 In our studies, we found that galectin-8 is required for targeting Mtb to selective autophagy.  
414 However, removal of this danger sensor did not completely abrogate targeting. This parallels what we  
415 have seen when the DNA sensor cGAS is depleted; around 50% of Mtb bacilli are still targeted (Watson  
416 et al., 2015). There are several possible explanations for this. First, these two pathways may function in  
417 parallel, each targeting some fraction of Mtb bacilli, adding up to the total of ~30% Mtb targeted in a wild-  
418 type cell. Future studies in cells lacking both cGAS/STING and galectin-8 could address this possibility.  
419 Second, it is possible that Rv1468c, Mtb's ubiquitin-binding surface protein, contributes substantially to  
420 the ubiquitin cloud, and because of Rv1468c, removing host sensors will only ever decrease targeting to  
421 ~15% (Chai et al., 2019). It is likely that some of the Rv1468c-bound ubiquitin chains serve as substrates  
422 to recruit TAX1BP1 and other adapters, so using Rv1468c mutants in future studies of host sensing  
423 pathways will help elucidate if additional host factors remain to be discovered in the targeting of Mtb.  
424 Ultimately, understanding the molecular mechanisms underpinning the host-pathogen interactions  
425 between this sort of Mtb protein and macrophage proteins will be critical for understanding the innate  
426 immune response to Mtb.

427 Previous studies of galectins have examined the *in vivo* requirement for individual galectins during  
428 Mtb infection. Interestingly, they have found that galectin-8<sup>-/-</sup> and galectin-3<sup>-/-</sup> mice succumb more rapidly  
429 to Mtb infection, suggesting these galectins are required for controlling Mtb infection (Chauhan et al.,  
430 2016, p. 3; Jia et al., 2018). However, these studies did not further interrogate how galectins contributed  
431 to innate immunity during Mtb infection, and galectins are multifunctional proteins that play a multitude of  
432 roles *in vivo* beyond their intracellular function in macrophages. To really understand how individual  
433 galectins contribute to macrophages' ability to control Mtb *in vivo*, future studies will need to infect mice  
434 with macrophage-specific deletions of these galectins. Such experiments would provide some of the best  
435 evidence to date of how selective autophagy in particular (rather than bulk autophagy) contributes to the  
436 control of Mtb infection *in vivo*. Of note, a previous study infected p62<sup>-/-</sup> mice with Mtb but found no

437 differences between wild-type and p62<sup>-/-</sup> mice; however, as demonstrated here and in other recent  
438 studies, several selective autophagy adapters are involved in detecting and targeting Mtb, so it is likely  
439 that removing multiple adapters will be necessary in order to study the *in vivo* requirement of selective  
440 autophagy adapters.

441 Finally, the finding that overexpression of galectins can enhance macrophages' ability to control  
442 Mtb is particularly noteworthy. Several host and bacterial factors can be mutated to diminish the targeting  
443 of Mtb to selective autophagy, but there are few known ways to enhance this targeting. In fact, for many  
444 other intracellular bacterial pathogens like Mtb, the targeted percentage is rarely above ~30%, suggesting  
445 this might be a biological setpoint that is difficult to overcome. However, it seems that galectin  
446 overexpression, even at the moderate levels permitted by our lentiviral expression system (Fig. S1B), is  
447 able to accomplish this. Identifying a class of proteins like galectins that can enhance targeting without  
448 causing significant off-target effects is extremely valuable in the future development of anti-TB therapies.  
449 For instance, overexpression or stimulation of cGAS, which is required for targeting, may enhance the  
450 number of targeted Mtb bacilli, but chronic activation of cGAS also results in enhanced production of type  
451 I IFNs, which are pro-bacterial and cause increased disease pathology *in vivo*. While chronic  
452 overexpression of galectins can have detrimental effects (Vinik et al., 2015), using small molecules to  
453 augment the function of galectins specifically during infection might be an especially attractive strategy  
454 for the future development of host-directed therapies for TB.

455

## 456 **MATERIALS AND METHODS**

### 457 **Cell lines and cell culture**

458 RAW 264.7 cells (ATCC TIB-71) and HEK293T cells (ATCC CRL-3216) were cultured in DMEM  
459 + 10% heat inactivated FBS + HEPES at 37 C with 5% CO<sub>2</sub>. Lenti-X (Takara Bio) cells were used for  
460 producing lentiviral particles. Where necessary, RAW 264.7 cells were selected with and maintained in  
461 Puromycin (InvivoGen, 5 µg/ml), Blasticidin (InvivoGen, 5 µg/ml), G418/Geneticin (InvivoGen, 750 µg/ml),  
462 or Hygromycin B (Life Sciences, 100 µg/ml). For infections, antibiotics were omitted from culture media.

463 RAW 264.7 cells were plated at  $2 \times 10^5$  cells/well in on circular glass coverslips in 24-well tissue culture  
464 (TC) dishes for immunofluorescence experiments and at  $3 \times 10^5$  cells/well in 12-well TC dishes for  
465 luciferase growth assays.

466 Epitope-tagged expression constructs were made by first cloning cDNAs from RAW 264.7 cell  
467 RNA into pENTR1a entry vectors with indicated tags (Addgene Plasmid #17396)(Campeau et al., 2009;  
468 Hoffpauir et al., 2020, p. 14; Watson et al., 2015). Constructs were fully Sanger sequenced (Eton  
469 Biosceinces, San Diego, CA) to verify the tagged proteins were complete, in-frame, and error-free.  
470 Constructs were then Gateway cloned with LR Clonase (Invitrogen) into pLenti destination vectors  
471 (Addgene Plasmid #19067)(Campeau et al., 2009). Expression of tagged proteins was confirmed by  
472 transfecting HEK293Ts with 1  $\mu$ g of pDEST and harvesting cell lysates after 1-2 days of expression.  
473 Proteins were separated by SDS-PAGE and visualized by Western blot analysis using primary antibodies  
474 for FLAG (Clone M2, Sigma-Aldrich, F1804) and HA (Roche, 11867423001).

475 To make RAW 264.7 stable expression cells lines, Lenti-X 293T cells (Takara Bio) were co-  
476 transfected with pLenti plasmids and the packaging plasmids psPAX2 and pMD2G/VSV-G (Addgene  
477 Plasmids #12259-60) to produce lentiviral particles. RAW 264.7 cells were transduced with lentivirus for  
478 two consecutive days plus 1:1000 Lipofectamine 2000 (Invitrogen) and selected for 3-5 days with  
479 antibiotic. Expression of tagged proteins was confirmed by Western blot analysis with antibodies against  
480 indicated tag.

481

## 482 **Bacterial infections**

483 Erdman was used as the parental Mtb strain for these studies (Stanley et al., 2007; Watson et al.,  
484 2012, 2015). The wild-type mCherry,  $\Delta$ esat-6 mCherry, and luxBCADE strains have been described  
485 previously (Budzik et al., 2020; Hoffpauir et al., 2020; Penn et al., 2018; Watson et al., 2012, 2015). Mtb  
486 cultures were grown in Middlebrook 7H9 (BD Biosciences) + 10% BBL Middlebrook OADC (Becton  
487 Dickinson) + 0.5% glycerol + 0.1% Tween-80 at 37°C in roller bottles. Strains were propagated with  
488 minimal passage.

489 Mtb infections were performed as previously described (Hoffpauir et al., 2020; Stanley et al., 2007;  
490 Watson et al., 2015). Briefly, cultures grown to 0.6-0.8 OD<sub>600</sub> were spun at 500g for 5 min to remove large  
491 clumps and then spun again at 3000g for 5 min to pellet bacteria. After washing twice with PBS, bacteria  
492 were resuspended in PBS, sonicated briefly to disrupt clumps, and then spun once more at 500g for 5  
493 min to remove remaining clumps. The OD<sub>600</sub> of the bacterial suspension was used to calculate the volume  
494 needed for the desired multiplicity of infection (MOI) of 1 (1 OD = 3x10<sup>8</sup> bacteria/ml). Bacteria were diluted  
495 in DMEM + 10% horse serum and added to cells. Infections were synchronized by spinning for 10 min at  
496 1000g, and cells were washed twice with PBS and cultured in regular media. When experiments lasted  
497 for more than 24 h, cell culture media was replaced daily. For IF experiments, at the indicated time points,  
498 coverslips were transferred to 4% fresh paraformaldehyde in PBS, fixed for 20 min, and washed three  
499 times with PBS. For luciferase experiments, cells were washed twice with PBS, lysed in 0.5% Triton X-  
500 100, and transferred to a white luminescence plate (LumiTrac 96-well plates, Greiner Bio-One).  
501 Luminescence was measured using a Tecan Infinite 200 PRO. For 0 h time point, cells were lysed after  
502 PBS washes rather than being returned to cell culture media.

503 *Listeria monocytogenes* infections were also performed as previously described (Watson et al.,  
504 2015). RAW 264.7 cells were plated at 1x10<sup>8</sup> cells per plate in 10 cm dishes. *Listeria monocytogenes*  
505  $\Delta$ actA (parental strain 10403, gift from Dan Portnoy) was grown in BHI (BD) at 30°C overnight without  
506 shaking. Culture was diluted 1:10 in BHI and grown for 3-4 h at 37°C without shaking until it reached an  
507 OD<sub>600</sub> of ~0.6. Bacteria were washed twice with HBSS, and the OD of the resulting bacterial suspension  
508 was used to calculate the volume needed for an MOI of 5 (1 OD=1x10<sup>8</sup> bacteria/ml). Bacteria were diluted  
509 in HBSS and added to cells. After incubating cells and bacteria for 30 min at 37°C, cells were washed  
510 twice with HBSS + 40  $\mu$ g/ml gentamycin and then cultured in media + 10  $\mu$ g/ml gentamycin until harvest.

511

## 512 **CRISPR/Cas9 Knockouts**

513 RAW 264.7 cells stably expressing FL-Cas9 were generated by transducing RAW 264.7 cells with  
514 lentivirus containing LentiCas9-Blast (Addgene plasmid #52962)(Sanjana et al., 2014). These cells were

515 selected with 5  $\mu$ g/ml Blasticidin (Invivogen) for 3-5 days and then with 10  $\mu$ g/ml Blasticidin for an  
516 additional 1-2 days. FL-Cas9 expression was confirmed via western blot analysis.

517 sgRNAs for each galectin gene were designed using the sgRNA Designer: CRISPRko website  
518 (<https://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design>) website and synthesized by  
519 IDT (Doench et al., 2016; Sanson et al., 2018). sgRNAs used for each galectin were as follows: gfp-1:  
520 gggcgaggagctgttcaccg; gfp-2: cagggtcagcttgccgtagg; gal3-1: tctggaaacccaaaccctca; gal3-2:  
521 ggctggttccccatgcacc; gal8-1: tcagtaatggtgccaacata; gal8-2: cagtaatggtgccaacatag; gal9-1:  
522 taccctccttctcaaaccg; gal9-2: acccccggttgaggaagga. Primers were cloned into LentiGuide-Puro  
523 (Addgene plasmid #52963) by phosphorylating, annealing, and ligating primers into digested vector  
524 (Sanjana et al., 2014; Shalem et al., 2014). sgRNA plasmids were validated by Sanger sequencing using  
525 the universal pLKO.1/hU6 promoter primer (Eton Biosciences, San Diego, CA). Lentivirus with sgRNAs  
526 were produced and used to transduce low passage FL-Cas9 RAW 264.7 cells. After selection with 5  
527  $\mu$ g/ml puromycin, the knockout efficiency was assessed at the population level. Using cells from the two  
528 most efficient sgRNAs, individual cells were serially diluted and plated into 96 well dishes to isolate clonal  
529 populations. When clones grew, populations were expanded, and each was assayed for mutations by  
530 amplifying a 500bp segment of genomic DNA around the mutation. These PCR fragments were Sanger  
531 sequenced using nested primers and compared to controls using TIDE analysis  
532 (<https://tide.deskgen.com>). Clones with homozygous nonsense mutations were further validated by  
533 measuring galectin RNA expression.

534 Triple knockout lines were made using a modified multiplexed lentiviral sgRNA system (Kabadi et  
535 al., 2014). The Cas9 in the lentiviral plasmid from this system was replaced with the puromycin resistance  
536 gene from a pDEST plasmid, which allowed for drug selection of a sgRNA array in RAW 264.7 cells  
537 already expressing FL-Cas9. sgRNAs for individual galectin genes were cloned into the sgRNA  
538 expression plasmids and assembled via Golden Gate assembly into the lentiviral backbone as previous  
539 published (Kabadi et al., 2014). RAW 264.7 cells expressing FL-Cas9 were transduced with lentivirus

540 containing sgRNA arrays (GFP sgRNAs or galectin sgRNAs), and cells were selected, cloned, and  
541 screened as above.

542

### 543 **Immunofluorescence**

544 Coverslips with fixed cells were blocked and permeabilized in 5% non-fat milk in PBS + 0.1%  
545 saponin for 30 min. Coverslips were then stained with primary antibody diluted in PBS with 5% milk and  
546 0.1% saponin for 2-4 h. Primary antibodies used in this study were FLAG (Clone M2, Sigma-Aldrich,  
547 F1804; 1:1000), FLAG (Sigma-Aldrich, F7425; 1:1000), HA (Roche, 11867423001; 1:1000), LC3  
548 (Invitrogen, L10382; 1:250), ubiquitin (Clone FK2, Millipore Sigma, 04-263; 1:500), p62 (Bethyl, A302-  
549 855A; 1:500), TAX1BP1 (A303-791A; 1:500), and OPTN (Bethyl, A301-829A; 1:500). Coverslips were  
550 washed three times in PBS and stained with secondary antibodies (Goat anti-Rabbit Alexa Fluor 488,  
551 Goat anti-Rat Alexa Fluor 647, and/or Goat anti-Mouse Alexa Fluor 647; Invitrogen, 1:1000) and DAPI  
552 (1:10,000) in PBS + 5% milk + 0.1% saponin for 1-2 h. Coverslips were then washed twice with PBS and  
553 twice with water and mounted using Prolong Gold Antifade Mountant (Thermo Fisher). Cells were imaged  
554 on an Olympus Fluoview FV3000 Confocal Laser Scanning microscope. Three coverslips per genotype  
555 were imaged, and at least 300 bacteria per coverslip were assessed and counted.

556

### 557 **Immunoprecipitations**

558 HEK293T cells were plated at  $5 \times 10^7$  cells per plate in 6cm TC dishes. The following day, cells  
559 were transfected with 2-5  $\mu\text{g}$  of indicated expression plasmids using PolyJet (SignaGen) according to  
560 manufacturer's instructions. Typically, 1  $\mu\text{g}$  of bait plasmid and 1-4  $\mu\text{g}$  of prey plasmid were co-  
561 transfected. After two days, cells were washed with PBS, lifted using PBS + EDTA, and pelleted by  
562 centrifuging at 3000g for 5 min. Cells were lysed in lysis buffer (150 mM Tris pH 7.5, 50 mM NaCl, 1 mM  
563 EDTA, 0.075% NP-40, protease inhibitors), and lysates were cleared of cellular debris and nuclei by  
564 spinning at 7000g for 10 min. 5% of the cleared lysate was saved as the "whole cell lysate", mixed with  
565 4x Laemmli sample buffer with fresh  $\beta$ -mercaptoethanol (Bio-Rad), and boiled for 5 min. Remaining cell

566 lysate was incubated with pre-washed (three times in 1 ml lysis buffer) 20  $\mu$ l of antibody-conjugated  
567 beads/resin (FLAG: EZview Red ANTI-FLAG M2 Affinity Gel, Sigma-Aldrich; HA: Pierce Anti-HA  
568 Agarose, Thermo Scientific) for 30-60 min at 4°C with rotation. Beads were washed three times with 1 ml  
569 wash buffer (150 mM Tris pH 7.5, 50 mM NaCl, 1 mM EDTA, 0.05% NP-40), and proteins were eluted  
570 with an excess of FLAG peptide (Sigma) or HA peptide (Sigma) resuspended in lysis buffer + 1% NP-40.  
571 Eluates were mixed with 4x sample buffer and boiled for 5 min. Proteins in whole cell lysates and  
572 immunoprecipitations were resolved by SDS-PAGE and imaged by western blot analysis using FLAG or  
573 HA antibodies (1:5000 in Li-Cor TBS Blocking Buffer), corresponding Li-Cor secondary antibodies  
574 (1:15,000), and a Li-Cor Odyssey Fc imager. Immunoprecipitations in RAW 264.7 cells stably expressing  
575 3xFLAG-tagged proteins were performed using the same protocol and workflow using cells infected with  
576 *Listeria monocytogenes* 1 or 2 h post-infection.

577

#### 578 **RNA extraction and RT-qPCR**

579 Cells were harvested in Trizol and RNA was extracted using Direct-Zol RNA MiniPrep kits (Zymo  
580 Research) with at least 1 h of on-column DNase treatment. cDNA was made using iScript (Bio-Rad), and  
581 gene expression was quantified using relative standard curves on a QuantStudio 6 Flex (Applied  
582 Biosystems) with PowerUp SYBR Green Master Mix (Applied Biosystems). Primers for *Actb* (F-  
583 ggtgtgatggtgggaatgg, R-gccctcgtcacccacatagga), *Lgals3* (F-ctggaaacccaaaccctcaa, R-  
584 aggagctgtcctgggtag), *Lgals8* (F-ccctatgttggcaccattact, R-gctgaaagtcaacctggaatct), and *Lgals9* (F-  
585 gccagctcctacattaacc, R-gttctgaaagttcaccacaaacc) were synthesized by IDT.

586

#### 587 **Exosomes**

588 RAW 264.7 cells were plated at  $5 \times 10^7$  cells per plate in 10 cm dishes. After 1 or 2 days in culture,  
589 cell culture media was collected, and cells were washed once with PBS and harvested by scraping. For  
590 whole cell lysates (WCLs), cells were pelleted and lysed directly in 1x Laemmli sample buffer with fresh  
591  $\beta$ -mercaptoethanol (Bio-Rad), sonicated to break up DNA, and boiled for 5 min. Culture media was pre-



592 cleared of dead cells and cell debris by spinning for 5 min at 3000g. Exosomes were then collected by  
593 ultracentrifugation for 1 h at 100,000g. Exosome pellets were resuspended directly in 1x sample buffer  
594 and boiled for 5 min. Proteins from WCLs and exosomes were resolved and imaged by SDS-PAGE and  
595 western blot analysis as described above using antibodies for FLAG (Sigma, F-1804; 1:5000), Alix  
596 (Abcam, ab117600; 1:2500), and syntenin-1 (Abcam, ab19903; 1:2500).

597

## 598 **Data analysis and presentation**

599 Statistical analysis was performed using Prism (GraphPad) with Student's unpaired two-tail t-  
600 tests. Graphs were generated with Prism, figures with Adobe Illustrator and Photoshop, and diagrams  
601 and schematics with BioRender.com as indicated in figure legends. At least three independent  
602 experiments were performed, and data presented is representative of these experiments. Figure legends  
603 indicate whether error bars indicate standard deviation (S.D.) or standard error of the mean (S.E.M.).

604

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613

## 614 **AUTHOR CONTRIBUTIONS**

615 Conceptualization, R.O.W., S.L.B., and K.L.P.; Investigation, S.L.B., R.O.W., K.L.L.; Writing, S.L.B.,  
616 K.L.P., and R.O.W.; Visualization, S.L.B. and R.O.W.; Funding acquisition, R.O.W., K.L.P., and J.S.C.;  
617 Supervision, S.L.B., R.O.W., K.L.P., and J.S.C.

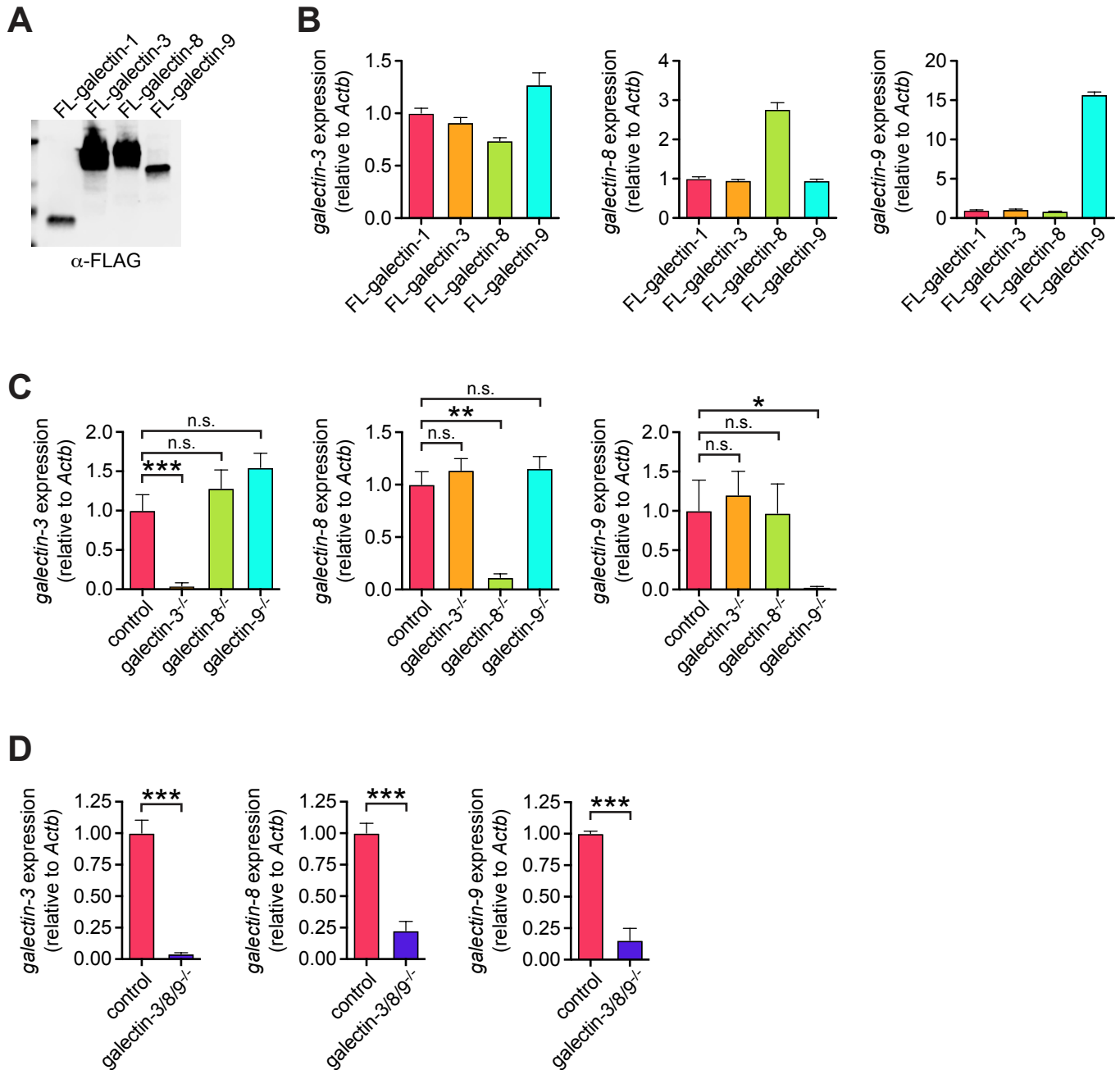
618

619 **CONFLICTS OF INTEREST**

620 The authors declare that the research described herein was conducted in the absence of any commercial  
621 or financial relationships that could be considered a conflict of interest.

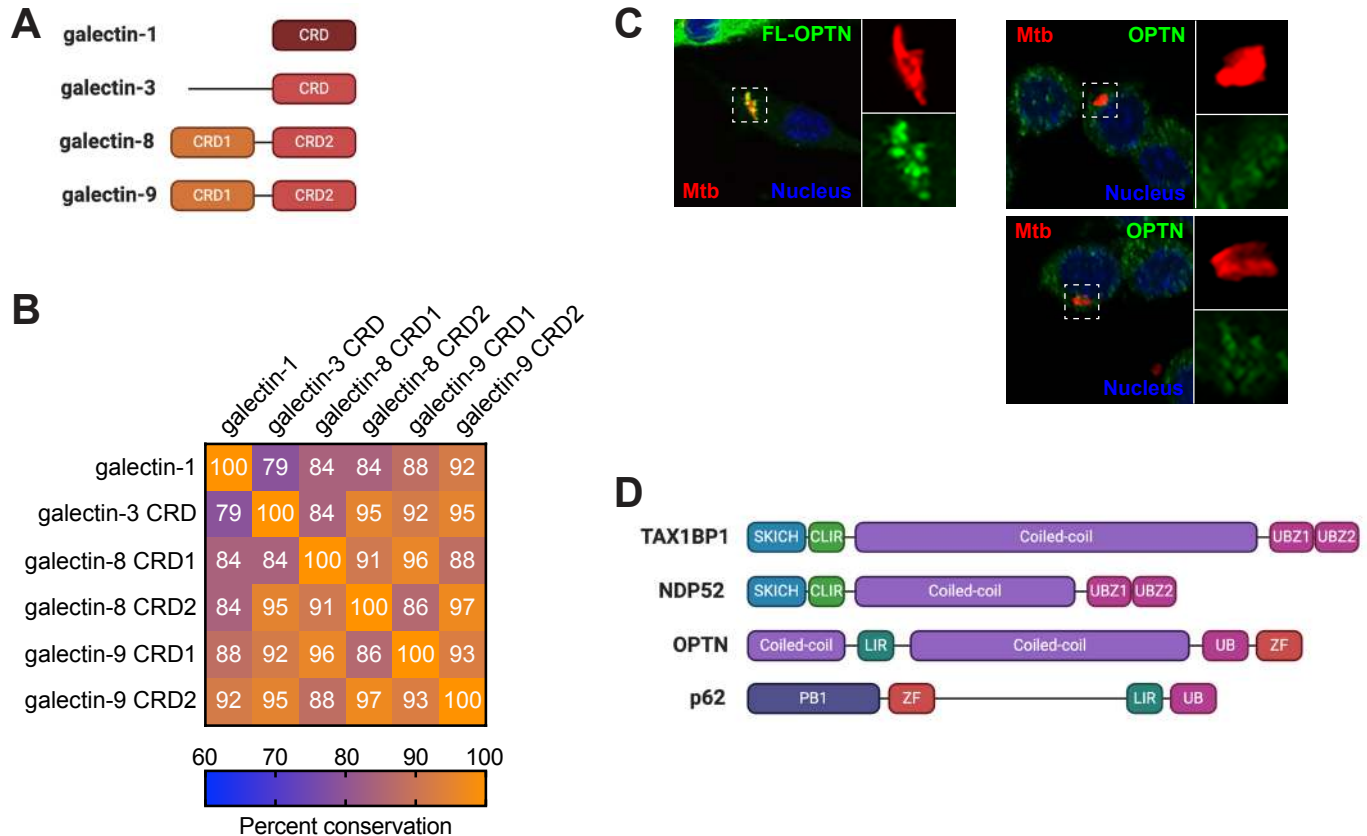
622

## Figure S1



**Figure S1. Validation of galectin stable expression and knockout cell lines.** (A) Western blot of whole cell lysates from RAW 264.7 cells stably expressing indicated 3xFLAG-tagged galectins. (B) Transcript levels of indicated galectins in cells stably expressing epitope-tagged galectins in RAW 264.7 cells. (C-D) As in (B) but for control (GFP gRNA) and individual knockout cell lines (C) or control (GFP gRNA) and triple knockout cell lines (D). Error bars are S.E.M. of the averages of each of 3-5 clonal cell lines of each genotype. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.005$ ; n.s., not significant.

## Figure S2



**Figure S2. Domains of galectins and adapter proteins.** (A) Schematic of the domains of galectin-1, -3, -8, and -9. CRD, carbohydrate recognition domain. (B) Percent conservation in pairwise comparisons of CRDs by analysis using M-Coffee. (C) Immunofluorescence of 3xFLAG-tagged OPTN (left) and endogenous OPTN (right) in RAW 264.7 cells infected with mCherry Mtb at 6 h post-infection. (D) Domain structure of selective autophagy adapters in this study. SKICH, coiled-coil, and PB1 are protein-protein interaction domains. CLIR, noncanonical/LC3C-interacting region; LIR, LC3-interacting region; ZF, zinc finger; UB, ubiquitin-recognition domain; UBZ, ubiquitin-binding zinc finger domain. Panels (A) and (D) made with BioRender.com.

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