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Supplemental Data

The Group A *Streptococcus* M1T1 clone subverts autophagy for intracellular replication

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SUPPLEMENTAL FIGURE LEGENDS

Figure S1, related to Figure 2. M1T1^{5448} GAS replicate efficiently in the cytosol of epithelial cells.

Association of intracellular M1T1^{5448} with EEA1/Lamp1 at 2 h and 6 h post-infection. Cells were simultaneously stained with mouse anti-EEA1 and mouse anti-Lamp1 primary antibodies and then with AlexaFluor-546-labeled goat anti-mouse secondary antibodies. Arrows indicate intracellular M1T1^{5448} associated with EEA1/Lamp1 at 2 h post-infection and intracellular M1T1^{5448} devoid of EEA1/Lamp1 at 6 h post-infection. Bar = 5 μm.

Figure S2, related to Figure 4. SpeB cysteine protease is required for efficient intracellular replication of GAS.

(A) Western immunoblot showing SpeB expression by different GAS strains used in this study. The zymogen (proSpeB) and mature (SpeB) forms of SpeB are indicated to the right.

(B) Survival curves after subcutaneous infection of humanized plasminogen transgenic mice (n = 10) with GAS strains M1T1^{5448} (4.5 x 10^7 colony forming units (CFU) per dose; dashed line) and M6^{JRS4} (9.1 x 10^7 CFU/dose; solid line). $p < 0.0001$, Log-rank (Mantel-Cox) test. Mice were infected as described previously (Cole et al., 2006).

(C) Percentage of intracellular GAS contained within LC3-positive compartments at 6 h post-infection. Values represent the mean ± SEM for three technical replicates. **, $p < 0.01$; one-tailed unpaired *t*-test.

(D) Ability of M6^{MGAS10394}, M12^{HKU16} and M4^{NS244} GAS to survive following internalization into HEp-2 epithelial cells. GAS strains were added to HEp-2 cells at a multiplicity of infection = 0.8. Extracellular GAS were killed by addition of DMEM containing 100 μg/ml gentamicin at 2 h post-infection. GAS viability was determined by direct plating of cell lysates and is represented as mean ± SEM of three independent experiments.

(E) SDS-PAGE of M1T1^{5448} culture supernatant and the purified SpeB preparation. The zymogen and mature forms of SpeB are indicated to the right. SpeB was the only bacterial protein identified in the purified SpeB preparation as determined by LC-ESI-MS/MS as described (Bailey et al., 2012).

(F) Purified SpeB cysteine protease degrades ubiquitylated proteins. SpeB was activated with 20 mM DTT and incubated with HEp-2 cell lysates in PBS for 120 min. A duplicate sample was incubated with 28 μM E64 to inhibit

SpeB activity. Immunoblots were probed with anti-ubiquitin monoclonal antibody P4D1 (Cell Signaling Technology). The size of molecular mass standards (in kilodaltons) is indicated. The arrow indicates the size of mono-ubiquitin. The GAPDH loading control was taken from Fig. 4e and is included for comparison.

(G) SpeB cysteine protease in M1T1^{5448} culture supernatants degrades ubiquitylated proteins and ubiquitin-LC3 adaptor proteins. Overnight cultures of each GAS strain were activated with 20 mM DTT and incubated with HEp-2 cell lysates in PBS for 120 min (ubiquitylated proteins) or 60 min (ubiquitin-LC3 adaptor proteins). A duplicate M1T1^{5448} sample was incubated with 28 μM E64 to inhibit SpeB activity. Immunoblots were probed with anti-ubiquitin monoclonal antibody. The size of molecular mass standards (in kilodaltons) is indicated. The arrow indicates the size of mono-ubiquitin (Ub).

(H to J) Representative images used for the quantitation of ubiquitin-LC3 adaptor proteins p62 (A), NDP52 (B) and NBR1 (C) following transfection of plasmids encoding human codon-optimised wild type SpeB (SpeB wt) and catalytically-inactive C192S SpeB (SpeB C192S). Bar = 5 μm.

Figure S3. Comparative genomic analysis of M1T1^{5448} and M1T1^{5448} Δ*speB*.

(A) *In vitro* growth of M1T1^{5448} and M1T1^{5448} Δ*speB* in THY broth *in vitro*. Each strain was cultured in THY broth at 37˚C and growth monitored by measuring OD_{600}. Values represent the mean ± SEM of three biological replicates.

(B) Circular genome map of the M1T1 reference strain MGAS5005 (Maamary et al., 2012) with BlastN comparisons to the draft M1T1 genome sequences of M1T1^{5448} (orange) and M1T1^{5448} Δ*speB* genomes (blue). GC content and GC skew of MGAS5005 is represented by the inner rings. M1T1^{5448} and M1T1^{5448} Δ*speB* differ by only 2 SNPs, located within the hypothetical protein MGAS5005_Spy_0895 (G -> T; Val -> Phe) and the glucan 1,6-alpha-glucosidase gene *dexB,* (G -> T; Asp -> Glu), which are not known to affect GAS virulence or gene expression.

(C) Genomic architecture of the *speB* genomic region of MGAS5005, M1T1^{5448} and M1T1^{5448} Δ*speB*. Red indicates regions of high sequence conservation as determined by Blast. The yellow open reading frame corresponds to the *speB* gene, with the blue open reading frame indicating the chloramphenicol (*cat*) antibiotic resistance marker. Integration site of the *cat* gene is indicated below the genomic comparisons showing precise allelic replacement. Black bars indicate stop codon sequences and the arrow indicates predicted start codons.

Table S1. Association of M1T1^{5448} and M6^{JRS4} with LC3, ubiquitylated proteins and Ub-LC3 adaptor proteins. Related to Figure 3.

	M1T1^{5448}				M6^{JRS4}			
	2h	4h	6h	8h	2h	4h	6h	8h
Percentage of intracellular GAS associated with LC3[a]	7.5 ± 1.6	3.1 ± 3.1	3.2 ± 0.9	1.1 ± 1.1	23.3 ± 10.1	38.6 ± 13.6	67.0 ± 2.0	32.5 ± 3.6
Percentage of LC3-positive GAS associated with[b]:								
Ub (FK2)	nd[c]	nd	nd	nd	82.5 ± 9.0	86.5 ± 3.2	82.5 ± 0.9	85.0 ± 1.2
p62	nd	nd	nd	nd	82.5 ± 3.7	96.3 ± 3.7	86.2 ± 0.1	95.0 ± 0.4
NDP52	nd	nd	nd	nd	13.7 ± 3.0	20.7 ± 1.3	29.4 ± 2.9	33.3 ± 4.3
NBR1	nd	nd	nd	nd	92.2 ± 4.0	98.4 ± 1.6	95.3 ± 0.9	98.1 ± 1.9

a Values are represented as mean ± s.e.m. of three independent experiments.

b Values are represented as mean ± s.e.m. of two independent experiments.

c nd, not determined as there were very low numbers of GFP-LC3-positive M1T1 GAS at all time points examined.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

GAS virulence assays

The virulence of GAS strains M1T1^{5448} and M6JRS4 was determined as described previously (Cole et al., 2006).

Comparative Genome Sequencing

The genomes of M1T1^{5448} and M1T1^{5448} Δ*speB* were sequenced using the Illumina MiSeq platform commercially at the Australian Genome Research Facility. 150 base paired-end reads were mapped onto the recently resequenced (Maamary et al., 2012) MGAS5005 M1T1 reference genome using SMALT (ftp://ftp.sanger.ac.uk/pub4/resources/software/smalt). The strains were sequenced to a mean coverage of 944-fold (M1T1^{5448}) and 1170-fold (M1T1^{5448} Δ*speB*). High-quality SNPs were identified with SAMtools mpileup and filtered with a minimum mapping quality of 30 and quality ratio cutoff of 0.75. The Illumina genome sequence data of M1T1^{5448} and M1T1^{5448} Δ*speB* were deposited into the ENA (http://www.ebi.ac.uk/ena/) under the accession numbers ERS351322 and ERS351323 respectively. A genome map of M1T1^{5448} and M1T1^{5448} Δ*speB* in context of the MGAS5005 M1T1 reference genome was determined by Blast comparisons using BRIG (Alikhan et al., 2011). *De novo* draft genome assemblies were compiled from 629,139 paired-reads based on a predicted coverage of 100x -fold using Velvet (Zerbino and Birney, 2008). The genome architecture of the *speB* loci was determined by BlastN analysis of draft genome assemblies using EasyFig (Sullivan et al., 2011).

SUPPLEMENTAL REFERENCES

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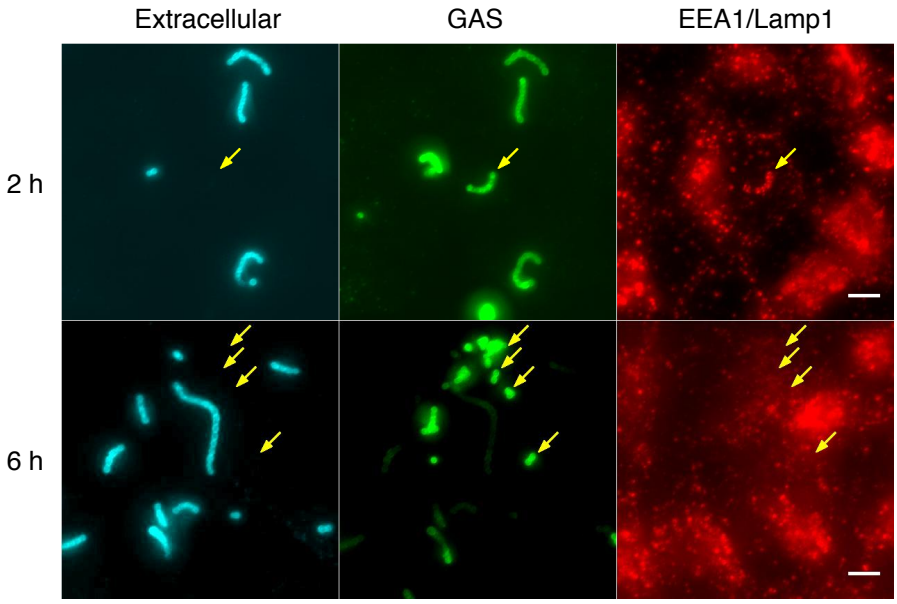
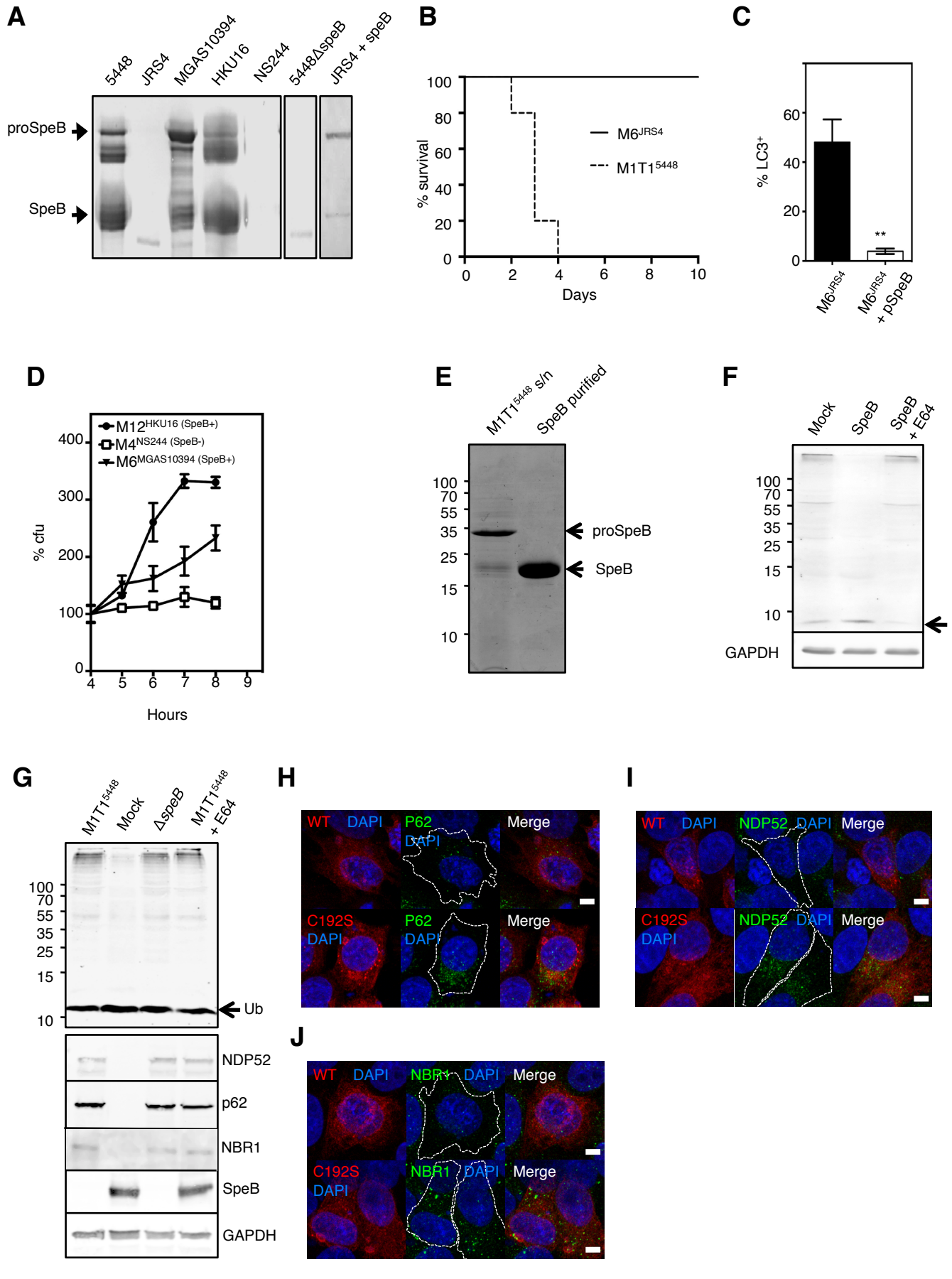
SUPPLEMENTARY FIGURES



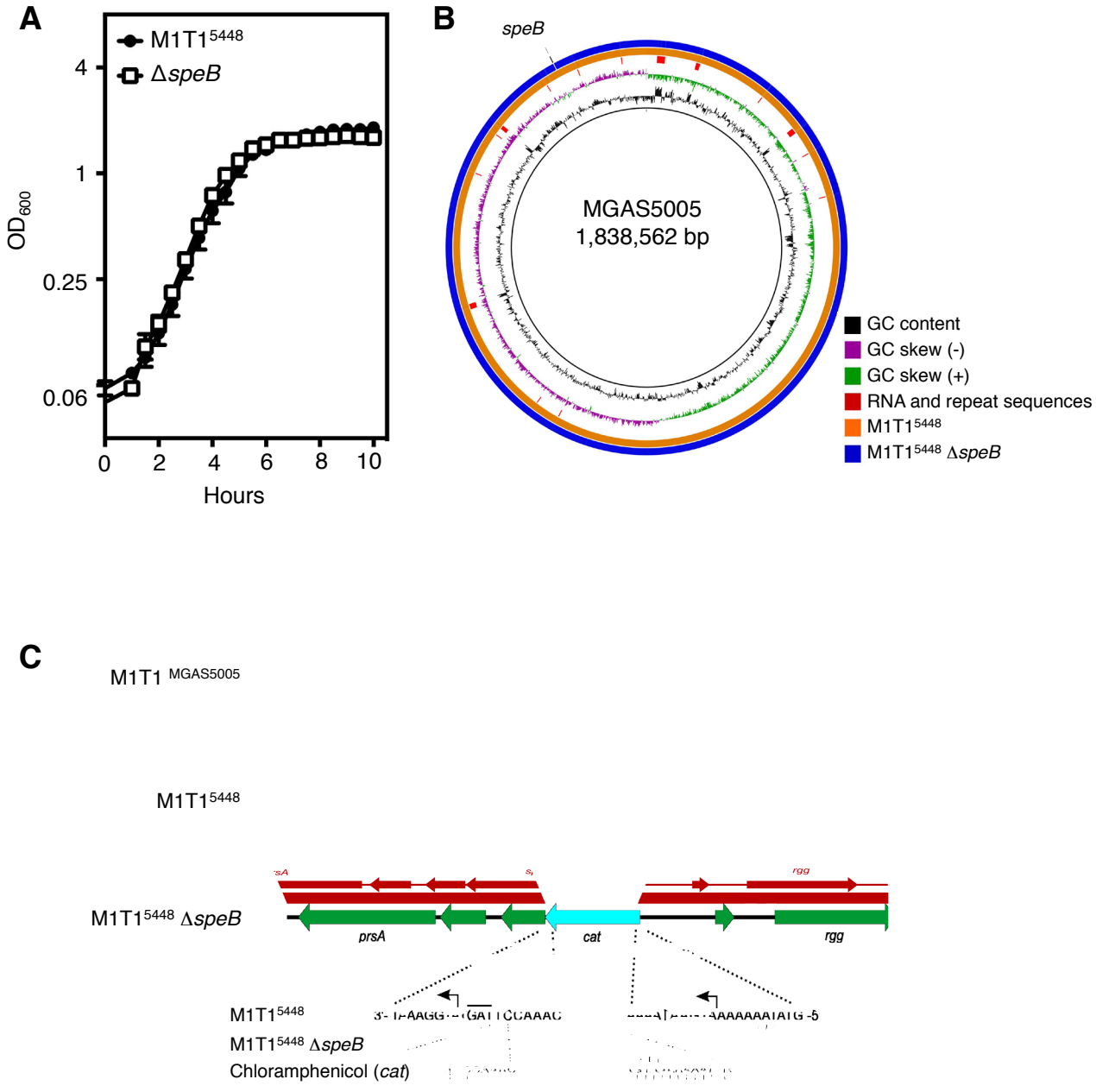
Barnett et al. Figure S1

Supplemental Figure 2



Barnett et al. Figure S2

Supplemental Figure 3



Barnett et al. Figure S3