

Cognate sigma and anti-sigma factors, and their relation to factors of the stressosome pathway in *Mycobacterium marinum*

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Abstract

Bacteria respond to environmental or physiological stimuli with rapid transcriptome switches involving alternate sigma (σ) factors released from inhibitory complexes with anti- σ factors. We have identified 11 genes corresponding to σ /anti- σ pairs in the fish pathogen *Mycobacterium marinum* and explored their mRNA levels during exponential growth as well as when subjected to several induced stresses. Co-immunoprecipitation and BACTH assays indicated that RscA (anti- σ^C), anti- σ^{2997} and anti- σ^{3276} interact with σ^C/σ^{C-1} , σ^{2997} and σ^{3276} , respectively. Together with validated data for *Mycobacterium tuberculosis* σ /anti- σ pairs suggest that *M. marinum* encodes a total of 10 anti- σ factors with anti- σ^C interacting with both σ^C and σ^{C-1} resulting in 11 σ /anti- σ pairs. We also provide RNA-Seq data suggesting similar mRNA levels (with a few exceptions) under different growth conditions and that the mRNA half-lives for σ and anti- σ factors in *M. marinum* were similar with the exception of the σ^L /anti- σ^L and σ^M /anti- σ^M pairs, but shorter than half-lives reported for *M. tuberculosis* and *Mycobacterium smegmatis*. We also argue that σ^{4487} and σ^{3687} should be assigned to the ECF41 σ -type while σ^G might constitute a group of its own but related to ECF41. We finally provide evidence that RskA (anti- σ^K) interacts with the stressosome pathway component RsbUVW indicating the presence of a new potential pathway where RsbUVW prevents RskA from binding to and inhibiting σ^K activity.

Introduction

Mycobacteria have evolved different strategies for rapid adaptations to changing environments, where the most common and potent is the alteration of transcription profiles in response to metabolic or environmental stimuli. One major way that bacteria use to modulate their global gene expression pattern in response to different environmental stimuli is to switch regulons using different sigma factors. Sigma (σ) factors are interchangeable subunits of RNA polymerase required to guide the polymerase to the promoters of genes to be transcribed. The different σ factors recognize specific promoter sequences and thus confer specificity upon the RNA polymerase holoenzyme for the promoters of a specific set of genes sharing similar promoter sequences¹⁻⁴.

All bacteria encode at least one essential σ factor to transcribe the housekeeping genes and at least one additional alternative σ factor (possibly with a few exceptions, e.g., some *Mycoplasma* spp.⁵; however, see⁶), which would allow rapid transcriptional shift by changing the recognition specificity of RNA polymerase for promoters of genes required for adaptation to altered metabolic and environmental conditions. The number of alternative σ factors usually reflect the complexity of the life cycle of the organism and the diversity of external or internal stimuli it can accommodate.

The slow growing fish pathogen *Mycobacterium marinum*, *Mmar* (a model system for *Mycobacterium tuberculosis*, *Mtb*⁷⁻¹⁰), encodes for 17-18 (dependent on strain) alternative σ factors^{11,12} attesting to its broad adaptive abilities to environmental changes. The presence of multiple σ factors require that their intracellular levels and activities be sensitive to changing environments or stages of growth. The expression of σ factors are regulated at the transcription and translation levels but the stability of the transcripts and proteins contribute to the intracellular levels of different σ factors. In addition, the activities of many σ factors are regulated at the post translational level^{1,4,13-18}. Among those, several of the ECF (extra-cytoplasmic function) σ factors are activated in response to stimuli originating from the extra-cytoplasmic environment, such as membrane damage or heat stress^{3,4}. These σ factors are ordinarily sequestered in an inactive state in a 1:1 complex with cognate anti- σ

factors resulting in blocking the σ factors' ability to activate the core RNA polymerase for transcription of specific sets of genes^{1-4,19-21}. Environmental stimuli result in degradation or a conformational change of the anti- σ factor thereby releasing and activating the σ factor^{14,19-21}. For example, regulated intramembrane proteolysis^{12-14,20,21} releases σ^K , σ^L , and σ^M (3 of 7 known σ /anti- σ factor pairs in *Mtb*) from their respective membrane bound anti- σ factors resulting in their activation²². Alternative modes of activation also exist such as partner-switching and protein-protein interaction cascades¹⁵. For instance, the general stress response σ factor, σ^B , in *Bacillus subtilis* is regulated through a phosphor-transfer cascade initiated through sensing of a stress signal by a multi-protein complex called the stressosome²³⁻²⁵. This cascade leads to sequestration of the anti- σ factor RsbW by the anti-anti- σ factor RsbV resulting in release and activation of σ^B . A similar pathway exists in many mycobacteria, in particular among slow growing mycobacteria (SGM), where the signal-induced phosphorylation cascade is suggested to lead to the activation of σ^F , the mycobacterial ortholog of *B. subtilis* σ^B ^{24,26,27}.

As for other bacteria, the activities of several σ factors in mycobacteria are regulated by anti- σ factors. Some of the anti- σ factors have, however, only been identified on the basis of bioinformatics^{13,16-18}, e.g., anti- σ^C and anti- σ^G in *Mtb* and all but two anti- σ factors, anti- σ^F (RsbW) and anti- σ^K (RskA), in *Mmar*²⁶⁻²⁸. Since anti-sigma factors control the activity of several mycobacterial σ factors, which are key factors in reprogramming the cell to adapt to changes in its environment, it is important to better understand how the cell coordinates this adaptation and σ /anti- σ factor interaction. We therefore decided to provide experimental evidence for their identity by demonstrating that the anti- σ factor candidates indeed interact with their cognate σ factors. Hence, we used bioinformatics to identify putative anti- σ factors in the *Mmar* genome and tested their interaction with their respective σ factors experimentally. To get insight into their expression we studied changes in σ and anti- σ factor transcript levels after exposure to various stresses and determined their mRNA stabilities using RNA-Seq. In addition, we tested whether any of the anti- σ factors, apart from RsbW, interacts with proteins of the stressosome pathway.

We provide data suggesting that anti- σ^C , anti- σ^{2997} and anti- σ^{3276} interact with their cognate σ factor, where anti- σ^C interacts both with σ^C and σ^{C-1} and that the anti- σ factor RskA interacts with the RsbUVW stressosome pathway protein. Moreover, our RNA-Seq data suggest similar transcript levels under different growth conditions (with a few exceptions) and that the mRNA half-lives for σ and anti- σ factors in *M. marinum* are similar with the exception of the σ^L /anti- σ^L and σ^M /anti- σ^M pairs, but shorter than half-lives reported for *Mtb* and *Mycobacterium smegmatis*.

Results

Identification of anti- σ factors and analysis of their gene synteny

Bacterial anti- σ factors regulate the activities of a number of σ factors and they consist of specific domains such as σ -binding and sensory domains^{see above e.g. 3,4,17,18,21} (Fig. 1 and below). As in other bacteria, anti- σ factor genes in mycobacteria are frequently located next to (usually downstream of) the genes encoding the σ factor they regulate. Therefore, to get insight into mycobacterial σ /anti- σ factors we performed a BLASTp search

using the protein sequences encoded by genes located next to the annotated σ factor genes of *Mmar*^M (M strain¹²) as outlined in Materials and Methods. Following this strategy, we identified two new candidates encoded by the open reading frames (ORFs) MMAR_2996 and MMAR_3277 (designated anti- σ ²⁹⁹⁷ and anti- σ ³²⁷⁶, respectively; not shown). The *Mmar* ORFs corresponding to Rv0181c (RsgA or anti- σ ^G) and Rv0093c (RscA or anti- σ ^C), predicted as anti- σ factors in *Mtb*^{13,14}, were also identified using the BLASTp approach. The *Mmar* gene corresponding to the putative *Mtb* RsgA (anti- σ ^G) homolog, MMAR_0424, is separated from *sigG* by MMAR_0425 and was rejected as an anti- σ factor candidate using our criteria. Also, MMAR_0424 is annotated as quercetin 2,3-dioxygenase in *Mmar* (Accession ID: WP_012392402.1) and in other mycobacteria (revealed by a BLASTp search against the NCBI nr database; non-redundant proteins, see Materials and Methods) supporting that it does not encode RsgA (anti- σ ^G). The annotated anti- σ factors to σ ^D, σ ^E, σ ^H, σ ^K, σ ^L, σ ^M, and σ ^F in the *Mmar*^M genome RsdA, RseA, RshA, RskA, RslA, RsmA, and RsbW, respectively, were also considered. We emphasize that these anti- σ factors have been experimentally verified in *Mtb*^{14–16}, which is phylogenetically close to *Mmar*^{29–32}. The corresponding proteins were $\geq 97\%$ identical in *Mmar*^{CCUG} and *Mmar*^{NCTC} (not shown; note that the σ and anti- σ factor genes are also present in the *Mmar* strains 1218R and 1218S^{33,34} whereas the σ ²⁹⁹⁷ and anti- σ ²⁹⁹⁷ genes are missing in the *Mmar*^{CCUG} strain¹⁰, and in *Mmar*^{ATCC927}, see Table S1f in Ref 33). Below we focus on *Mmar*^{CCUG 33,34}, and the gene identities for the σ and anti- σ factors in *Mmar*^{CCUG} (and *Mtb*^{H37Rv}) are given in Table S1.

Intracellular anti-sigma factors generally consist of a single anti-sigma factor domain (ASD), but they can also exist as intramolecular inhibiting domains of sigma factors. Membrane bound anti-sigma factors have an intracellular domain (the ASD), a transmembrane domain, and sometimes an extracellular domain¹⁵. Thus, the known and putative anti- σ factors were analyzed for the presence of conserved domains using the Conserved Domain Database (CDD) and InterProScan^{35,36}. Both approaches predicted putative domains, present in previously characterized anti- σ factors, in all candidates and known anti- σ factors except RseA (anti- σ ^E), RsmA (anti- σ ^M) and anti- σ ²⁹⁹⁷. Using CDD (but not InterProScan) predicted anti- σ factor domains in RseA (anti- σ ^E) and anti- σ ²⁹⁹⁷ while neither CDD nor InterProScan predicted any anti- σ factor domains in RsmA (anti- σ ^M; Fig. 1; Table S2).

The zinc finger zf-HC2 domain was the most common followed by the anti- σ factor RsrA domain (antisig_RsrA). The zf-HC2 is a subdomain of antisig_RsrA and therefore only zf-HC2 is shown in Fig. 1 (but, see Fig S1 and below). Furthermore, RscA (anti- σ ^C), RsdA (anti- σ ^D), RskA (anti- σ ^K), RslA (anti- σ ^L), and RsmA (anti- σ ^M) were predicted to have transmembrane domains suggesting they might be membrane bound (Table S2). In parallel, we analyzed the corresponding anti- σ factors in *Mtb* H37Rv with similar results except that no transmembrane domain was detected in *Mtb* RsmA (anti- σ ^M; Table S2).

The gene synteny for the anti- σ factors are conserved in the three *Mmar* strains, *Mmar*^{CCUG}, *Mmar*^{NCTC} and *Mmar*^M (and in *Mmar*^{1218R}, *Mmar*^{1218S} and *Mmar*^{ATCC927})^{11,32,33,34} and *Mtb* (Fig. 2) with the exception for the *sigH/rshA* and *sigC/rscA* pairs. In *Mtb*, a conserved hypothetical gene separates *sigH* and *rshA*, while they are positioned next to each other in *Mmar*. (The ORFs of the three *Mtb* genes each overlap by four nucleotides perhaps indicating that the conserved hypothetical protein is functionally related to σ ^H/RshA but we have no evidence for this.) The second difference relates to the *sigC/rscA* synteny (Fig. 2). In *Mtb*, *rscA* is positioned at a distant location on the chromosome relative to *sigC*. *Mmar* has two *sigC* genes, *sigC* and *sigC_1*, where the

single anti- σ^C gene (*rscA*) is separated from *sigC_1* by an ORF encoded on the opposite strand. The gene synteny upstream of *rscA* is conserved as revealed by comparing *Mmar* and *Mtb*. The second *sigC* is located distant from *rscA* and *sigC_1* with a similar gene synteny as in *Mtb*. However, *rscA* and *sigC* are transcribed in opposite directions in *Mtb*, while they are positioned in the same orientation in *Mmar*. Thus, a chromosomal rearrangement might have occurred in either of the species. In this context, no repeated sequences or IS element is present in *Mmar* while *Mtb* carries a repeated sequence belonging to the REP13E12 family close to *rscA*^{37,38}.

The gene syntenies for the σ^{2997} /anti- σ^{2997} and σ^{3276} /anti- σ^{3276} pairs are conserved in the *Mmar* M, NCTC, 1218R and 1218S strains (both pairs are absent in *Mtb*)^{33,34}. Since the σ^{2997} /anti- σ^{2997} pair is missing in the *Mmar*^{CCUG} and *Mmar*^{ATCC927} strains (see above and also^{11,33}) indicates that the pair is not essential for growth under standard laboratory conditions. A BLASTp search further indicated that anti- σ^{2997} is more common among SGM compared to anti- σ^{3276} (Supplementary Table S3).

Analysis of the domain structures of putative redox controlled anti- σ factors

Many of the ECF anti- σ factors contained the zf-HC2 motif (6 ECF anti- σ factors in *Mmar* and 4 in *Mtb*; Table S2), and are therefore putative redox responsive anti- σ factors. To analyze this the conserved Cys/HisX₂₃₋₂₆HisX₃CysX₂Cys³⁹ (where X_n is n number of any amino acids and the Cys/His are involved in coordinating Zn²⁺) zinc-associated anti- σ factor (ZAS) motif was identified. RslA and RseA from *Mtb* and *Mmar* both have the HisX₂₄HisX₃CysX₂Cys-motif in common with the archetypal ZAS anti- σ factor ChrR³⁹ (Fig S1), while the remaining four (two in *Mtb*) ZAS anti- σ factors have the CysX₂₅HisX₃CysX₂Cys-motif in common with the other archetypal ZAS anti- σ factor RsrA³⁹ (Fig S1). The ZAS anti- σ factor secondary structures were predicted using JPred 4⁴⁰ and compared with ChrR and RsrA (Fig S1). The three alpha-helical domains (Helix 1–3, boxed in Fig S1) positioning the Zn²⁺-coordinating residues of the zf-HC2-motif (residues labeled in yellow; Fig S1) are conserved in all proteins. JPred 4 predicted helix 4 to be present in four of the *Mmar* ZAS anti- σ factors. However, helix 4 was not predicted to be present in RslA, neither in *Mmar* nor in *Mtb*, despite that a previous report indicated its presence in *Mtb* RslA⁴¹. On the basis of this and the weak sequence similarity in the region comparing *Mmar* and *Mtb* RslA we cannot exclude that helix 4 is present in *Mmar* RslA.

Bioinformatics-based identification of domains within σ factors that conceivably act as intramolecular "anti- σ factors"

Some σ factors have a C-terminal domain involved in regulation of σ factor activity in *cis*^{4,14,see also 17,18}. Therefore, we analyzed all σ factors for the presence of such a domain using CDD and InterProScan (see Materials and Methods). The *Mmar* σ^G , σ^J , σ^{3687} , and σ^{4487} , and *Mtb* σ^G , σ^I , and σ^J were identified to have a similar domain architecture as the *Bacillus licheniformis* and *Rhodococcus sphaeroides* σ factors encoded by the BLi04371 and RSP_0607 genes, respectively¹⁴. They all have a " σ_{70} region 2 like" and a " σ_{70} region 4 like" domain followed by a C-terminal extension similar to an NTF2-like domain (Nuclear transport factor). Since both BLi04371 and RSP_0607 encode a domain that inhibits the σ factor activity unless activated¹⁴, it is likely that

this is also the case for these *Mmar* and *Mtb* σ factors (see above). A reciprocal blast hit approach where we searched for the presence of the NTF2-like domains from BLi04371 and RSP_0607 in the protein coding sequences of *Mmar* and *Mtb*, and *vice versa* provided support for this suggestion. For *Mmar* σ^J and σ^{4487} and *Mtb* σ^J , we obtained reciprocal BLASTp hits with RSP_0607, and for *Mmar* σ^{4487} with BLi04371 (Table 1). A unidirectional hit was obtained for *Mtb* σ^I with RSP_0607 while neither σ^G (*Mmar* and *Mtb*) nor σ^{3687} produced any BLASTp hits. However, we obtained σ^J as a reciprocal hit for σ^{3687} (and for σ^{4487}) by performing an interspecies BLASTp search with the NTF2-like domains indicating a relationship. It is pertinent to note that these ECF σ factors were among those for which no "traditional" anti- σ factor gene was identified (Figs. 1 and 2; Table S2). Below we focus on the bioinformatically identified (and known) σ -/anti- σ factor pairs.

Table 1
Presence and location of NTF2 domains in mycobacterial σ factors

Species	Protein	Length	σ^{70} R2	σ^{70} R3/4	NTF2	BLI04371	RSP_0607
<i>B. licheniformis</i>	BLI04371	285	2–82	87–156	169–280	Nt, Nt	$2e^{-8}$, $6e^{-8}$
<i>R. sphaeroides</i>	RSP_0607	286	6–91	101–155	166–284	$6e^{-8}$, $2e^{-8}$	Nt, Nt
<i>Mmar</i>	σ^G	343	30–104	151–206	219–341	Nd, Nd	Nd, Nd
<i>Mmar</i>	σ^J	309	11–89	111–168	168–299	Nd, Nd	$2e^{-8}$, $2e^{-7}$
<i>Mmar</i>	MMAR_3687	299	8–86	109–167	179–289	Nd [#] , Nd [#]	Nd [#] , Nd [#]
<i>Mmar</i>	MMAR_4487	301	11–91	88–169	174–293	$1e^{-10}$, $2e^{-11}$	$2e^{-15}$, $1e^{-16}$
<i>Mtb</i>	σ^G	370	56–131	178–234	235–366	Nd, Nd	Nd, Nd
<i>Mtb</i>	σ^I	290	9–87	95–165	173–283	Nd, Nd	Nd, 0.80
<i>Mtb</i>	σ^J	312	6–82	100–156	168–291	Nd, Nd	$2e^{-5}$, $6e^{-4}$
<i>Msmeg</i>	σ^G	340	24–100	117–202	214–338	Nd, Nd	Nd, Nd
<i>Msmeg</i>	σ^J	304	4–86	105–161	167–292	Nd, Nd	$8e^{-5}$, $3e^{-4}$
<i>Msmeg</i>	MSMEG_1692	304	19–82	119–171	172–269	$8e^{-8}$, $7e^{-6}$	0.029, $5e^{-4}$
<i>Msmeg</i>	MSMEG_5444	301	7–93	105–166	176–296	Nd, Nd	$5e^{-12}$, $8e^{-13}$
<i>Msmeg</i>	MSMEG_6817	296	11–91	86–164	172–292	Nd, Nd	$1e^{-8}$, $3e^{-10}$
Positions and lengths in amino acids. σ^{70} R2 = σ factor region 2 and σ^{70} R3/4 = σ factor regions 3 and 4 ⁵² .							
NTF2 = Nuclear transport factor 2-like domain. BLI04371 and RSP_0607: forward and reverse BLASTp e-values with the NTF2-like domain of the respective protein are given with the forward BLASTp e-values first. Nt: not tested. Nd: no detected hits. [#] A reciprocal BLASTp hit with <i>Mmar</i> σ^J was detected.							

Interaction between the putative anti- σ factors and their cognate σ factors

To understand if the putative anti- σ factors and their corresponding σ factors (discussed above) indeed interact we used two approaches, the bacterial two-hybrid system (BACTH using full-length proteins, see also Supplementary information for BACTH details and a schematic representation of the BACTH technology, Fig S2; note, that BACTH can give false positives)^{42,43} and co-immunoprecipitation (Co-IP). The σ^M /RsmA and σ^D /RsdA pairs (for *Mtb*, these σ /anti- σ pairs have been reported to interact⁴⁴) served as controls to ensure specific interactions and we tested σ^C , σ^{C-1} , σ^{2997} , and σ^{3276} with RscA, anti- σ^{2997} , and anti- σ^{3276} in all possible combinations. The results were scored as outlined in Materials and Methods and Supplementary methods. Pertaining to Co-IP (discussed below), we used size separation and any unspecific signals should appear at the same positions on the blots and gels for all the samples and since no such signals were detected indicating absence of unspecific interactions (see also the controls in Figs. 3 and 6).

As shown in Figs. 3A and S3 all tested cognate σ /anti- σ pairs produced blue single colonies indicating their ability to interact (BACTH assay). Only a few weak interactions for non-cognate pairs (σ^{3276} /anti- σ^{2997} and possibly σ^C /anti- σ^{3276}) were detected (not shown) indicating specificity of cognate interactions.

Next, we co-expressed the cognate σ /anti- σ pairs from a common promoter to establish that they were expressed in a 1:1 ratio. The anti- σ factor in each pair was tagged with a 6xHis tag and the respective σ factor with a FLAG peptide tag. The 6xHis-tagged anti- σ factors were then purified using a HisPur cobalt resin to identify interacting proteins (or anti-6xHis antibody/protein-G agarose for RscA_{cyto} co-expressed with σ^{C-1} , see Supplementary Methods for details). For σ^C , we used both the σ^C /RscA and σ^C /RscA_{cyto} pairs since, as discussed above, RscA is predicted to carry a transmembrane region (see also below). After separation by SDS-PAGE and staining with Oriole gel stain, two distinct bands of different sizes were detected corresponding to the sizes of the respective σ - and anti- σ factor (except for the σ^C /RscA, probably because the RscA with its transmembrane region remained in the insoluble membrane fraction; Fig. 3B, lanes 3 and 8, and not shown). To verify that the detected signals corresponded to the σ and anti- σ factors, replicate Western blots were prepared and probed with antibodies targeting the 6xHis (anti- σ factors) and FLAG peptide (σ factors) tags (Fig. 3C; the seemingly weaker anti- σ protein signals relative to the σ protein signals might be related to differences in antigenicity of the His and FLAG tags but at present we do not have any explanation to this difference but see⁴⁵). These results confirmed that the tested (see above) cognate σ factors co-purified with their corresponding anti- σ factor and provided evidence supporting that they interact. For unknown reasons, RscA_{cyto} could not be detected by Western blotting. Nevertheless, the stained SDS-PAGE gel showed a distinct band migrating at the expected position and molecular mass of RscA_{cyto} (Fig. 3B, lanes 4 and 9; see also below). Thus, the co-purification of σ^C provides tentative support for an interaction between σ^C and RscA_{cyto}.

To test the σ^{C-1} /RscA_{cyto} interaction, we modified the Co-IP protocol used for the σ^C /RscA_{cyto} pair as described in Supplementary Methods. The results showed that both σ^{C-1} and RscA_{cyto} could clearly be detected by Western blot and SDS-PAGE (Fig. 3D, E). The 6xHis tagged RscA_{cyto} pulled down FLAG tagged σ^{C-1} , suggesting a direct interaction between RscA_{cyto} and σ^{C-1} (cf. Figure 3D, lane 1 and 3E, lane 1). Importantly, the 6xHis tagged RscA_{cyto} did not pull down the FLAG tag alone (Fig. 3D, lane 2 and 3E, lane 2) indicating that the interaction is not due to non-specific binding between the tags. Moreover, the 6xHis tagged RscA_{cyto} also pulled down untagged σ^{C-1} (Fig. 3E, lane 3). We also tried a dot blot approach where both a FLAG-tag and a 6xHis-tag

specific antibody were first used to pull down the FLAG- and 6xHis-tagged proteins followed by immune-detection using the same primary antibodies in combination with fluorescently labeled secondary antibodies (Fig S4G-K). Together, these data suggest that σ^{C-1} /RscA_{cyto} interact.

We conclude that the tested *Mmar* σ and anti- σ factors interact with their predicted cognate partner and thereby consistent with that they constitute σ and anti- σ factor pairs.

Probing predicted anti- σ factor membrane topologies using BACTH data

To provide insight into the predicted membrane topology of the putative transmembrane anti- σ factors (RscA, RsdA, RskA, RslA and RsmA; in this analysis, we also included anti- σ^{2997} , and anti- σ^{3276}) we analyzed the BACTH data (see above). If the predictions based on bioinformatics are correct, an active adenylate cyclase should only be reconstituted when either of the adenylate cyclase domains is fused to the anti- σ factors predicted cytoplasmic terminus carrying the predicted cytoplasmic domain (see Supplementary information for details and Fig S2).

Our analysis supports the prediction for the four anti- σ factors RsdA, RskA, RslA and RsmA (see above; data for RscA, RsdA, RsmA, anti- σ^{2997} , and anti- σ^{3276} , see Fig S5, and RskA and RslA, see Fig S12). For RsdA, only the construct where RsdA was fused to the adenylate cyclase fragments at its N-terminal (cytoplasmic) side resulted in adenylate cyclase activity with the cytoplasmic σ^D fused to the complementary fragment (Fig S5). For RskA and RslA, the BACTH data were in agreement with their predicted membrane topology (see above and Fig S12) and for RscA, predicted to have both termini in the cytoplasm, active adenylate cyclase was reconstituted with fusions either at the N- or C-terminus. For RsmA, with a predicted cytoplasmic N-terminus, activity was detected for three of four constructs when the adenylate cyclase domain was fused to the RsmA N-terminus in agreement with our prediction (see also Supplementary information).

These data together with the bioinformatics predictions are consistent with that the *Mmar* anti- σ factors RsdA, RskA, RslA and RsmA are putative transmembrane proteins. Further experiments are required to conclusively establish that this indeed is the case. For RscA, the BACTH data are inconclusive but, as discussed above, the bioinformatics predicted the presence of transmembrane domains in RscA (Fig. 1).

Transcript levels of genes encoding σ and anti- σ factors

In general σ - and anti- σ factor genes are positioned next to each other in the genome (Fig. 2) and are therefore likely to be co-transcribed^{3,4,16,46}. Previously we reported the abundance of *Mmar* σ factor mRNA levels in cells growing exponentially, in stationary phase and under several stress conditions. For example, RNA-Seq and qRT-PCR data suggested that mRNA levels for σ^B and σ^E increased in stationary phase and during starvation while σ^C and σ^{C-1} decreased^{11,47}. To get insight into their expression and expand this analysis we used RNA-Seq to understand whether the mRNA levels of the *Mmar* anti- σ factors and their corresponding σ factors changed in a coordinated manner under the same growth and stress conditions. Biological replicate samples from exponential and stationary phases, microaerobic stress, heat and cold exposure, nitrous stress, membrane/antibiotic stress (isoniazid), DNA damage/SOS stress (mitomycin C), acidic stress, osmotic stress, oxidative stress, and starvation were analyzed. *Mmar* was exposed to the different stress conditions for 24

hours except in the case of microaerobic stress where we subjected the cells to oxygen depletion for 12 days (see Materials and Methods)¹¹. Experimental replicate reproducibility was verified using the Spearman correlation coefficient, which ranged from 0.76 to 0.94 (not shown). Moreover, exposure to different stresses influence growth differently as measured by OD₆₀₀¹¹ (see Supplementary information).

Comparing mRNA levels for the σ /anti- σ factor pairs showed high relative abundance for σ^E and anti- σ^E while the levels for σ^C , σ^{C-1} and RscA were significantly lower irrespective of growth and stress conditions (Fig. 4). Transcript levels for the other σ /anti- σ factor pairs were also lower in comparison to the σ^E /anti- σ^E pair but note that σ^D /anti- σ^D , σ^F /anti- σ^F and σ^H /anti- σ^H pairs showed some, albeit small, variations. Moreover, the σ /anti- σ factor pairs showed similar and parallel relative abundances under the conditions tested. But, for some pairs we did observe deviations when comparing mRNA levels (Figs. 5 and S6). In stationary phase the σ^C and σ^E mRNA levels were higher than the levels for RscA and RseA, respectively. In contrast, RscA (anti- σ^C) and RsmA (anti- σ^M) transcript levels increased in stationary cells relative to σ^{C-1} and σ^M , respectively. In response to different stresses we did observe deviations, in particular when comparing σ^C and RscA (e.g., cold and nitrous stress), σ^{C-1} and RscA (e.g., microaerobic stress), σ^E and RseA (anti- σ^E ; most notable during starvation), and σ^M and RsmA (anti- σ^M ; acidic, cold, microaerobic, nitrous and osmotic stress).

Together, it appears that the σ - and anti- σ factor genes are co-transcribed. This is supported by the paired end read data where we detected reads and read pairs stretching from within one of those genes, across the junction between them, and into the other gene (Figs S7 and S8). However, dependent on growth and stress conditions differences in relative mRNA levels might indicate regulation at the transcriptional level and/or differences in mRNA degradation (see below and the discussion).

σ and anti- σ factor mRNA levels over time

To detect transient shifts, if any, in transcription profiles following induction of stress (*i.e.*, before the organism adapted to the altered environment) we measured the σ /anti- σ factor mRNA levels as a function of time starting immediately after exposure to the stress conditions using qRT-PCR. In these experiments, we compared the σ /anti- σ factor mRNA levels for cells exposed to heat, cold, and microaerobic stress (Fig S9; the σ factor mRNA data was taken from¹¹). For microaerobic stress, the endpoint was 12 days while for heat and cold stress it was 24 hours (see figure legend 4).

Overall, the trajectory of the σ factor and anti- σ factor curves were similar for all stresses with two notable exceptions. Firstly, comparing σ^M and RsmA (anti- σ^M) transcripts during cold stress, σ^M increased while RsmA decreased relative to exponential phase. Secondly, the RscA (anti- σ^C) mRNA levels remained roughly unchanged with a possible increase after 24 hours while the level for σ^C decreased and σ^{C-1} increased followed by a decrease during cold stress. With these two exceptions, this close correlation between the σ and anti- σ factor mRNA levels as a function of time are in keeping with that they are co-transcribed (for correlation coefficients and p-values for changed mRNA levels for each time point compared to 0 h, see Table S4). For comparison, we included the mRNA levels for the housekeeping σ^A factor¹¹. In heat and cold stresses its mRNA level remained unchanged, while during microaerobic stress it decreased rapidly (Fig S9), similar to what has been reported for the other σ factors¹¹.

Taken together, the conservation of gene synteny, the presence of typical anti- σ factor domains, the observed interaction between the cognate σ /anti- σ factor pairs based on BACTH and pulldown/Co-IP data, and the close correlation between the mRNA levels for the anti- σ factor and corresponding σ factor provide evidence that the predicted proteins likely represent anti- σ factors in *Mmar*.

The mRNA stabilities of σ and anti- σ factors are similar

Since the σ /anti- σ factor pairs are frequently co-transcribed as a single transcript^{3,4,16,46} it is reasonable to expect their mRNA stabilities to be similar. Therefore, we decided to determine the half-lives for all σ and anti- σ factor mRNAs in *Mmar*^{CCUG} (see Materials and Methods).

The half-lives for the *Mmar* anti- σ factor mRNAs, except for RslA (5.1 min), ranged between 1.8 to 3.1 min (average 2.4 min; Table 2 and Fig S10). Similarly, the half-lives for the σ factor mRNAs ranged between 1.8 and 9.1 min (average 3.2 min). The σ^M mRNA had the longest half-life, 9.1 min, but with low confidence ($R^2 = 0.45$). We could detect a significant difference in half-lives between the σ factors lacking (average 3.6 min) and having (average 2.3 min) an anti- σ factor only after exclusion of σ^M ($P < 0.05$; Student's t-test, two tailed and unpaired). Pairwise comparison between the σ and anti- σ factor mRNA half-lives showed that they were within 0.8 min from each other except for σ^C /RscA (1.8 vs 3.1 min), σ^L /RslA (2.9 vs 5.1 min) and σ^M /RsmA (9.1 vs 2.8 min).

Table 2
Half-lives for σ and anti- σ factor mRNAs from three different mycobacteria

Gene	Half-life ^a (R ² -value)		
	<i>Mtb</i> H37Rv ^b	<i>Msmeg</i> MC2 155 ^b	<i>Mmar</i> CCUG 20998 ^c
<i>sigA</i>	6.2 (0.950)	4.6 (0.998)/1.2 (0.920) ^d	2.0 (1.000)/2.0 (0.940) ^d
<i>sigB</i>	8 (0.939)	7.7 (0.930) ^d	4.7 (0.897)/2.3 (0.882) ^d
<i>sigC</i>	7.2 (0.925)		1.8 (0.945)
<i>rscA</i>			3.1 (0.912)
<i>sigC_1</i>			2.3 (0.953)
<i>sigD</i>	7.8 (0.899)		2.1 (0.950)
<i>rsdA</i>	8.8 (0.891)	6.6 (0.862)	2.2 (0.958)
<i>sigE</i>	8.6 (0.901)		2.6 (0.927)
<i>rseA</i>			3.0 (0.906)
<i>sigF</i>	12.5 (0.913)		2.2 (0.958)
<i>rsbW</i>	10.4 (0.929)		1.8 (0.969)
<i>sigG</i>			2.1 (0.940)
<i>sigH</i>	8 (0.961)	6.8 (1.000)	2.2 (0.969)
<i>rshA</i>	8.5 (0.948)	7.6 (0.944)/1.6 (0.948) ^d	2.1 (0.971)/2.1 (0.995) ^d
<i>sigJ</i>		5.6 (0.908)	3.9 (0.771)
<i>sigK</i>	8.4 (0.947)		2.3 (0.935)
<i>rskA</i>	9.4 (0.926)		2.3 (0.924)
<i>sigL</i>		2.7 (0.808)	2.9 (0.884)
<i>rslA</i>		4.6 (0.825)	5.1 (0.704)
<i>sigL_2</i>		5.3 (0.982)	
<i>sigM</i>		4.9 (0.944)	9.1 (0.448)
<i>rsmA</i>			2.8 (0.847)
<i>sig0975</i>			5.1 (0.694)
<i>sig3276</i>			2.1 (0.943)

^aHalf-life in minutes. ^bValues from⁴⁷ estimated by microarray analysis except where noted with

superscript d. ^cValues estimated by transcriptome analysis (see Materials and Methods) except where noted with superscript d. ^dFrom qRT-PCR (this work).

Gene	Half-life ^a (R ² -value)		
	<i>Mtb</i> H37Rv ^b	<i>Msmeg</i> MC2 155 ^b	<i>Mmar</i> CCUG 20998 ^c
asig3276			2.1 (0.971)
sig3687			4.6 (0.745)
sig4487			2.7 (0.937)
MSMEG_1348		6.6 (0.989)	
MSMEG_4315		4.2 (0.996)	
<i>rsbR</i>			1.9 (0.977)
<i>rsbS</i>			2.0 (0.982)
<i>rsbT</i>			2.1 (0.955)
<i>rsbX</i>			2.3 (0.971)
<i>rsbV</i>	8.0 (0.953)		1.9 (0.964)
<i>rsbUW</i>			3.2 (0.872)
<i>rsbUVW</i>	12.9 (0.911)		2.0 (0.974)
^a Half-life in minutes. ^b Values from ⁴⁷ estimated by microarray analysis except where noted with			
superscript d. ^c Values estimated by transcriptome analysis (see Materials and Methods) except where noted with superscript d. ^d From qRT-PCR (this work).			

The half-lives of the *Mmar* mRNAs determined by qRT-PCR and RNA-Seq were considerably shorter than those of the corresponding mRNAs in *Mtb* and *M. smegmatis* (*Msmeg*), which were determined by microarray analysis⁴⁸ (Table 2). To test whether this was due to a true difference in the mRNA half-lives comparing these mycobacteria or related to the different techniques used, the half-lives for σ^A , σ^B , and RshA mRNAs in *Msmeg* were determined (Table 2). These data suggested that the microarray analysis resulted in longer half-lives compared to the qRT-PCR. Our limited testing indicates that comparing absolute mRNA half-lives generated with different techniques might therefore skew the interpretation of the data.

The RskA anti- σ factor is linked to the stressosome pathway

To test whether there is any link between anti- σ factors other than RsbW (anti- σ^F , see introduction) with proteins constituting the stressosome pathway all identified *Mmar* anti- σ factors were studied for their interaction with the RsbV, RsbUW, and RsbUVW proteins using the BACTH system and a Co-IP assays (see above, Materials and Methods, and Supplementary Methods; see also^{26,27}). RsbUVW is a multi-domain protein with functional RsbU (PAS and PP2C phosphatase), RsbV (STAS), and RsbW (kinase) domains fused into a single protein⁴⁹ unlike in *B. subtilis* where they are individual single-domain proteins. Similarly, RsbUW is a multi-domain protein with domains similar to both RsbU (RsbT binding domain, PP2C phosphatase domain) and RsbW (kinase), but it also has a PAS/PAC domain²⁴. In addition, *Mmar* encodes the single-domain RsbV and RsbW/anti- σ^F (but not RsbU)

proteins. The previously identified RsbV/RsbW interaction was included for comparison^{26,27} (Fig. 3C). Positive interactions were scored as outlined in Materials and Methods, and Supplementary Methods.

The BACTH data shown in Figs. 6A-D, S11 and S12 suggested that RskA interacts with RsbUVW given the intense blue color on "LA X-gal" plates. No other interactions could be reliably detected albeit weak and/or sporadic positive signals were observed, which might indicate weak interactions, in particular for RslA with RsbUW and RsbUVW. Nevertheless, since the RskA-RsbUVW interaction showed strong and consistent signals we decided to validate this interaction. Hence, we cloned RsbUVW with a 6xHis-tag and expressed it together with the cytoplasmic domain of RskA, referred to as RskA_{cyto}, tagged with the FLAG-tag. RskA_{cyto} is small (9.7 kD) and as in the case of RscA_{cyto} (see above) we were unable to detect RskA_{cyto} on a Western blot membrane, nor in the crude lysate indicating a low expression level and/or instability (not shown). Moreover, while RsbUVW could not be detected after pull-down with HisPur cobalt resin, 6xHis-tagged RsbUVW could easily be detected in the crude lysate using antibodies specific for the 6xHis-tag (not shown; see also below). We therefore attempted to Co-IP using the 6xHis-tag specific antibodies and protein-G agarose. Here, we also used anti-FLAG antibodies to Co-IP FLAG-tagged RskA_{cyto}. These results suggest a weak interaction or alternatively reflect low RskA_{cyto} levels (see Supplementary information, Fig S4G-K). Hence, we repeated the Co-IP assays in *E. coli* BL21 (DE3) pLysS cells, which improve protein yield (see Supplementary Methods). In these experiments, IPTG-uninduced cells served as controls. Western blot analysis show that RskA_{cyto}-FLAG (in the C-terminal) co-purified with both N- and C-terminal 6xHis-tagged RsbUVW variants (Fig. 6E) indicating that RsbUVW and RskA_{cyto} interact.

Together, these findings suggest that RskA interacts with RsbUVW and thereby expand the previously presented stressosome protein interaction network^{26,27} to include RskA and σ^K (Fig. 6F).

Discussion

Bacterial σ factors have essential roles for binding of RNA polymerase to promoters resulting in initiation of transcription. The activities for some of these are regulated at the post-translational level by their cognate anti- σ factors^{20,21}. The *Mmar*^{CCUG} and *Mmar*^{ATCC927} strains encode 17 σ factors while the *Mmar*^M, *Mmar*^{1218R} and *Mmar*^{1218S} strains have 18^{11,33,34}. The activities for ten (nine in *Mmar*^{CCUG} since it lacks σ^{2997} /anti- σ^{2997}) of these are potentially regulated through interaction with anti- σ factors. Our bioinformatics-, BACTH-, Co-IP- and RNA-Seq data support the notion that σ^C /RscA, σ^{C-1} /RscA, σ^D /RsdA, σ^M /RsmA, σ^{2997} /anti- σ^{2997} and σ^{3276} /anti- σ^{3276} represent σ /anti- σ factor pairs in *Mmar*. Among these, the σ^D /RsdA and σ^M /RsmA pairs, as well as σ^E /RseA, σ^F /RsbW(UsfX), σ^K /RskA and σ^L /RslA pairs, have been verified and reported to be present in *Mtb*^{16,22,41,50–52}. Together with our current data this would account for all σ /anti- σ factor pairs in *Mmar* and thereby expand our knowledge about σ /anti- σ factor pairs in mycobacteria. In this context, Manganeli¹⁶ predicted the presence of RscA in *Mtb* and considering that *Mmar* is phylogenetical close to *Mtb*^{29–31,32} we suggest that it also interacts with σ^C and regulates its activity in *Mtb* while in *Mmar* RscA interacts with both σ^C and σ^{C-1} .

Co-transcription and expression of σ /anti- σ factor genes

Our RNA-Seq data revealed that for most cognate σ and anti- σ factor pairs the mRNA level stoichiometry was roughly 1:1 irrespective of tested conditions with a few notable exceptions (see below). Also, the temporal σ and anti- σ factor mRNA levels in response to heat, cold, and microaerobic stresses correlated (Fig S9). This is in keeping with the side-by-side positioning of their genes in *Mmar* and in mycobacteria in general. That the anti- σ and the σ factor pairs are co-transcribed may contribute to maintenance of the 1:1 stoichiometry of the corresponding proteins^{1,3,4,15,19,53} thereby supporting the interrelation between the anti- σ factors and the corresponding cognate σ -factor (see above).

The *sigC* (and *sigC_1*) gene are present preferentially among slow growing mycobacteria such as *Mtb* and *Mmar* (Pettersson *et al.*, unpublished) and it has been implicated to have a role in pathogenesis^{54,55}. As discussed above, σ /anti- σ factor genes are positioned next to each other on the chromosome but this does not apply to *sigC*, *sigC_1* and *rscA* (anti- σ^C). The *sigC* is positioned $\approx$ 3.2 Mb distant from *rscA* ($\approx$ 2.2 Mb in *Mtb*) while *sigC_1* is separated by one gene from *rscA* and this gene (gene id: CCUG20998_00250; *copA_2*, copper-exporting P-type ATPase A) is transcribed in the opposite direction relative to *sigC_1* and *rscA* (*sigC_1* and *rscA* are transcribed in the same direction). This argues for that *sigC*, *sigC_1* and *rscA* are not being co-transcribed as appears to be the case for the other σ /anti- σ factor gene pairs. However, as for the other pairs the RNA-Seq data suggested that the *sigC*, *sigC_1* and *rscA* mRNA levels were similar (with a few notable exceptions). This raises questions about how these genes are regulated and whether their expression is coordinated. In *Mtb*, data suggest that *sigC* is transcribed by " σ^A RNA polymerase" from two promoters P1 and P2⁵⁵. A putative " σ^A -10 sequence" could be identified in *sigC*^{*Mmar*} at the position corresponding to the *Mtb* P1 promoter while a P2 promoter could not be detected (Fig S13). For *sigC_1*^{*Mmar*} (57.6% amino acid identity and 69.6% amino acid similarity compared to *Mmar* σ^C), we identified a putative " σ^C -10 sequence" overlapping the predicted translational start site GTG. But, an alternative potential ATG start site is located downstream of the GTG. A putative " σ^C -10 sequence" was also predicted for *rscA* in both *Mmar* and *Mtb* H37Rv. We therefore hypothesize that σ^C might be involved in transcribing *sigC_1* and *rscA* thereby linking the expression of these three genes. Further experiments are needed in order to demonstrate whether this is actually the case.

Apparent deviations from the 1:1 mRNA level stoichiometry (Figs. 5 and S6) was detected for some σ /anti- σ factor pairs, such as: σ^E /RseA(anti- σ^E) (stationary phase and starvation conditions) and σ^M /RsmA(anti- σ^M) (in stationary phase, and under conditions of acidic, cold, microaerobic, nitrous and osmotic stress). For σ^E /RseA, the distance between the translated region of *sigE* and *rseA* corresponds to 145 bases in *Mmar*, which is similar to the distance in *Mtb* H37Rv (157 bases; Fig. 2). Various *in vivo* and *in vitro* studies have provided insight into the expression and regulation of *Mtb sigE*. Three promoters upstream of *sigE* have been identified resulting in generation of three σ^E isoforms in *Mtb* H37Rv⁵⁶. These promoters are also present in *Mmar*. Surveying the *sigE*-*rseA* intergenic region revealed the presence of putative σ^E , σ^H and σ^F "-10 regions" as well as weak σ^H "-35 regions" in both *Mmar* and *Mtb* H37Rv. In addition, putative σ^E "-10 and -35 (weak) regions" upstream of the σ^E translational stop codon was identified in *Mmar* (Fig S14A; note, in mycobacteria -35 regions appear to be of less importance⁵⁷). Hence, this raises the possibility that *rseA* might be transcribed separately from *sigE* and thereby rationalize higher levels of *rseA* mRNA during starvation and in stationary phase. Indeed, pertaining to *Mtb* it has been reported that *rseA* is transcribed independently of *sigE*⁵⁸, and references therein. A small RNA, *mpr6*, is also transcribed from the intergenic region between *sigE* and *rseA* in, e.g. *Mtb* and *Mmar*. In *Mtb* and *Mmar*, the coding region for *mpr6* overlaps the 5'UTR and 22 *rseA* residues and it is transcribed in the same orientation

as the σ^E and *rseA* (Fig S14A)⁵⁹. The *mpr6* function is unknown but its transcription and presence (Fig S14B) raise the question whether it influences the level of either σ^E or *rseA* mRNA, and this remains to be studied.

The mRNA levels for the σ^M /RsmA pair deviated for several growth conditions (see above). Surveying the 75 nucleotides long *sigM-rsmA* intergenic region did not reveal any known mycobacterial promoter sequences⁵⁷ that could provide a rationale to these deviations. However, *sigM* and *rsmA* have been reported to be independently transcribed in *M. tuberculosis* and *M. smegmatis*^{60,61}. Nevertheless, albeit the lower confidence value for *sigM*, our data suggest a lower stability for the *rsmA* mRNA relative to *sigM*. This would explain the difference in mRNA levels in stationary cells and upon exposure to acidic, nitrous (nitric oxide) and osmotic stress, but not why σ^M mRNA is higher during in particular cold stress. But, lowering the temperature is known to stabilize RNA in bacteria in general⁶² and both cold and microaerobic stress reduce mRNA degradation in *Mtb* and thereby increase mRNA stability⁴⁸. Thus, the increased σ^M mRNA level might be the result of the interplay between mRNA stability and degradation⁶³. In this context, previous data suggest coupling between mRNA degradation and metabolic status in *Msmeg*⁶⁴. Moreover, the "mRNA stability-degradation interplay" might also apply to the σ^L /RslA pair for which the mRNA half-lives were determined to be 2.9 and 5.1 min, respectively. However, we did observe similar levels irrespective of growth and stress conditions suggesting involvement of additional regulatory circuits and these need to be identified.

The remaining σ /anti- σ factor gene pairs in *Mmar*, σ^D /RsdA, σ^F /RsbW, σ^H /RshA, σ^K /RskA and σ^{3276} /anti- σ^{3276} (Fig. 2) are closely linked and annotation suggests that the UGA stop codon for the σ -factors overlaps with the putative translational start of the anti- σ factors (note for σ^F /RsbW, *rsbW* precedes *sigF*). For σ^D /RsdA and σ^F /RsbW this also applies to *Mtb* H37Rv while for σ^K /RskA there is a 47 base pairs extension downstream of the σ^K UGA translational stop codon, which contains an AUG translation start codon (not shown). This is in keeping with co-transcription of these pairs and our findings that their mRNA stabilities were the same. Translation of the downstream anti- σ factors (and σ^F) appears therefore to be the result of frameshifting⁶⁵. Whether this has an impact on their expression and abundance needs to be investigated. In this context, a mass spectrometry analysis of *Mtb* H37Rv⁶⁶ peptides, corresponding to the N-terminus of Rv2537c and Rv1295, indicated that translational start sites, TSS, overlap with the translation stop codon of the annotated upstream gene. Peptide analysis of our unpublished mass spectrometry *Mmar*^{CCUG} data also revealed overlapping translational start (*pspA_3*, gene id: CCUG20998_03005) and termination sites (*uppP*, gene id: CCUG20998_03006). These data provide support to the occurrence of frameshifting during translation of σ^D /RsdA, σ^F /RsbW, σ^H /RshA, σ^K /RskA and σ^{3276} /anti- σ^{3276} but further experiments are required to conclusively demonstrate that this is the case.

The RsbRST stressosome and anti- σ factors

The stressosome is a multi-protein complex, composed of RsbR, RsbS and RsbT and referred to as RsbRST, involved in the bacterium's response to environmental and energetic stress. In *B. subtilis*, the stressosomal response manifests through the activation of σ^B , the general stress σ factor. Its activation involves a cascade of phosphotransferase and partner switching mechanisms involving, among other proteins, the anti- σ factor RsbW and the anti-anti- σ factor RsbV^{23–25}. In mycobacteria, the σ factor resembling *B. subtilis* σ^B is σ^F (in terms of gene synteny and protein similarity/domain composition), while the anti- σ factor is either referred to as RsbW or UsfX^{26,67,68}. σ^F and RsbW are found in most, if not all, mycobacteria, unlike the remaining components of the

stressosome activation pathway^{26,27}. *Mtb* lacks most of the pathway, including RsbV (but it has *rsfB/rv3687c*), but instead has the multi-domain RsbUVW (encoded by *rv1364c*) consisting of functional RsbU, RsbV, and RsbW domains (see above; also, we note that in *Msmeg* RsbW is deactivated by RsfA and RsfB⁶⁸ and we cannot exclude that they are present in *Mmar*, which would add to the number of RsbW antagonists, and possible other anti- σ factor antagonists in *Mmar*). RsbUVW has been suggested to interact both with σ^F and RsbW^{69,70}. *Mmar* on the other hand, harbors both a near complete stressosome pathway with separate RsbV and RsbW proteins and RsbUVW. In addition, it encodes RsbUW having both RsbU and RsbW domains. RsbUW and RsbUVW were previously shown to interact with the stressosome proteins RsbT and RsbV, but neither interacted with RsbW nor σ^F ²⁶. We therefore considered that RsbV, RsbUW and RsbUVW might interact with other anti- σ factors and our data (Figs. 6C, E and S4G-K) suggested that RsbUVW interacts with the RskA anti- σ factor. Presumably RsbUVW can act as an anti-anti- σ factor through its C-terminal STAS domain, which is common in anti-anti- σ factors⁷¹. RsbUVW, possessing a signal sensing PAS domain, may possibly directly sense an input signal⁷⁰, thus bypassing the RsbRST complex. Some of the other anti- σ factors resulted in less consistent and weaker signals when tested for interaction with stressosome factors. In particular, RslA showed interesting patterns of interactions with RsbUW and RsbUVW when the adenylate cyclase fragment was fused to the predicted intracellular domain of RslA. However, based on these data alone we cannot at present claim a positive interaction, but a true interaction cannot be excluded. Further experiments are needed to resolve this question.

Interestingly, RscA, RsdA, RskA, RslA, and RsmA were predicted to be membrane bound anti- σ factors and RskA, RslA, and RsmA can be deactivated through regulated intramembrane proteolysis by the Rip1 protease in *Mtb*²². Since RskA likely interacts with RsbUVW it is possible that RsbUVW may deactivate RskA through a different mechanism compared to Rip1. Conceivably, RsbUVW could prevent RskA mediated membrane anchoring of σ^K by blocking this interaction from the intracellular side. In this context, a positive interaction between RsbUVW and RskA was only detected when the adenylate cyclase domain was fused to the predicted intracellular domain of RskA (RskA_{cyto}) supporting the bioinformatics prediction of membrane topology. Thus σ^K activity could be induced under conditions where Rip1 is not active thereby expanding our understanding of the regulation of σ factor activity in mycobacteria. Since *Mtb* RskA has been shown to be redox sensitive²² another possibility is that the RsbUVW mediated deactivation of RskA is linked to redox stress. We also note that it has been reported that RskA has an activator-inhibitor dual function controlling the activity of SigK⁷². Whether this is linked to the RsbUVW interaction with RskA remains to be studied.

mRNA stabilities in mycobacteria

With a few notable exceptions, the σ factor and anti- σ factor of cognate σ /anti- σ factor pairs had similar mRNA half-lives in *Mmar* (see above). In contrast, the half-lives of the anti- σ factors in *Mtb* and *Msmeg* (for which only limited data are available⁴⁸) tended to be longer compared to their corresponding σ factors (Table 2).

Furthermore, the half-lives of the *Mmar*^{CCUG} σ and anti- σ factors were considerably shorter compared to those of both the slow growing *Mtb* and the rapid growing *Msmeg* (Table 2). Our limited testing of this possibility suggested that, at least partly, the method used to determine the half-lives influenced the estimated values, where qRT-PCR (this study) tended to result in shorter half-lives compared to those estimated by microarray (used for *Mtb* and *Msmeg*⁴⁸). This has been reported also to be the case for the slow-growing marine cyanobacteria *Prochlorococcus*⁷³. However, one study of mRNA decay in a *Halobacterium salinarum* strain reported that microarrays, Northern blots and qRT-PCR resulted in similar mRNA half-lives, but the number of

tested genes were limited⁷⁴. At present, we do not have any explanation to why the method (microarray vs qRT-PCR) used to determine mycobacterial mRNA half-lives influence the outcome. To understand this requires further experiments using a larger number of genes. In this context, and as discussed above, we emphasize that mRNA stability/degradation in bacteria depends on metabolic state^{63,64,75}.

Classification and type of σ factor regulation in mycobacteria

A new type of σ factor regulation was discovered where a C-terminal extension of the σ factor folds into an inhibitory conformation preventing the σ factor from participating in transcription¹⁴. These ECF σ factors were grouped into type ECF41 by Staroń *et al.*⁴⁶ (for updated ECF classification see^{17,18}). This type, characterized by having a σ^{70} region 2-like and a σ^{70} region 4-like domain followed by a C-terminal extension with similarity to an NTF2-like domain, has predominantly been found in *Proteobacteria* and *Actinobacteria*^{14,46}. Our results suggest that this type of σ factor domain structure is common among mycobacteria. Of the ECF σ factors lacking a corresponding anti- σ factor in *Mmar* the majority [4 out of 5, *i.e.*, σ^G , σ^J , σ^{3687} and σ^{4487} (Table 1); the exception being σ^{0975}] had this domain arrangement. These were either the σ^J or σ^J -like σ factors, including σ^I where present, or σ^G or σ^G -like σ factors. The *Mtb* σ^I and σ^J belong to the ECF41 type^{76,77}. The σ^G could not be assigned to any type⁴⁶ but it shares similar domain architecture as σ^I and σ^J . Two σ factors, *sigG1* and *sigG2*, in *Streptomyces tsukubaensis* show sequence homology with *Mtb* σ^G and *sigG1* belongs to ECF56⁷⁸. Hence, we argue that *Mmar* σ^{4487} and conceivably σ^{3687} should be assigned to ECF41, and that mycobacterial σ^G is related to ECF41 given the reciprocal BLASTp search results (Table 1) using the NTF2-like domains but likely belongs to ECF56. Furthermore, σ^J and σ^G commonly share the property of being up-regulated upon *Mtb* infecting macrophages. Also, σ^J was found to regulate σ^I ⁴⁹ and Refs therein,⁷⁶ and σ^I was recently found to be up-regulated upon macrophage infection by a smooth colony variant of *Mtb*⁷⁹. Thus, there appears to be a link between these σ factors in terms of their domain architecture and in their function further arguing their assignment to the same or a related type.

Materials and Methods

Bioinformatics and identification of the σ -/anti- σ factor pairs in *Mmar*

Previously suggested¹⁶ and Refs therein or annotated anti- σ factor sequences and their corresponding σ factor sequences were obtained from the *Mtb* H37Rv and *Mmar* strain M (*Mmar*^M) genomes. Since the anti- σ factors are usually encoded by sequences next to those of their respective σ factor, the neighboring genes of the remaining σ factors (unless the annotation clearly indicated a completely unrelated function) and if transcribed in the same orientation on the chromosome, were extracted together with the σ factor genes as putative σ /anti- σ pairs. The protein sequences encoded by these genes were analyzed using the BLASTp program of the BLAST package⁸⁰ by searching against the NCBI nr (non-redundant proteins) database with default settings (e-value threshold 10). A hit containing the word "anti- σ factor" or similar within the first 100 hits without any other hit with a specified function/name having a higher score, was considered an anti- σ factor candidate. Proteins fulfilling these criteria were identified in the *Mmar* CCUG 20998 (*Mmar*^{CCUG}) and NCTC 2275 (*Mmar*^{NCTC}) genomes^{11,34} by BLASTp searches against their annotated coding sequences. The putative anti- σ factor

sequences were analyzed for the presence of domains related to anti- σ factor function using the Conserved Domain Database batch search function (CDD version 3.15) with e-value cut-offs set at 0.01, 0.1, and 1.0, as well as the InterProScan database search function (version 58.0). Subsequently, their secondary structures were predicted using JPred 4⁴⁰ and compared with the structure of known anti- σ factors. The same approach (except for the secondary structure predictions) was used to analyze the σ factors. The gene synteny of the σ /anti- σ factor pairs were analyzed for the three *Mmar* genomes, strains *Mmar*^M, *Mmar*^{CCUG} and *Mmar*^{NCTC} (we also inspected the *Mmar*^{1218R}, *Mmar*^{1218S} and *Mmar*^{ATCC927} genomes^{32–34}) and the *Mtb* H37Rv genome, and the distances between the stop codon of the upstream gene and the start codon of the downstream gene were mapped. Positive values refer to that the reading frames of the two genes are separated by the number of nucleotides indicated, while negative values indicate the number of nucleotides by which the reading frames are overlapping.

Bacterial Two-Hybrid system and analysis of protein-protein interactions

Cloning of the σ and anti- σ factor genes into the BACTH vectors pKT25, pKNT25, pUT18, and pUT18C⁴², generating N-terminal and C-terminal protein fusions, was done following standard molecular biology protocols essentially as described in^{26,27} using genomic DNA from the *Mmar*^{CCUG} strain (except for the σ^{2997} /anti σ^{2997} pair where the source of the genomic DNA was *Mmar*^{NCTC}; the σ^{2997} /anti σ^{2997} gene region is missing in *Mmar*^{CCUG}¹¹). The cloning of the RsbV, RsbW, RsbUW, RsbUVW, and σ^F genes have been reported elsewhere²⁶. Pairs of putatively interacting proteins were analyzed essentially as previously described^{26,27}. For details, see Supplementary methods, and Fig S2.

Pertaining to Co-immunoprecipitation, pulldown, Co-purification of interacting proteins, Western blotting and Dot blotting, for detail method descriptions see Supplementary Methods.

Analysis of mRNA levels

Mmar^{CCUG} cells were grown and exposed to various stresses as previously described¹¹. RNA from duplicate samples was extracted and further processed to remove traces of DNA as previously described¹¹ and submitted for whole transcriptome sequencing (RNA-Seq) at the SNP&SEQ Technology Platform (HiSeq2000—Illumina—platform) at Uppsala University and at GATC Biotech (Konstanz, Germany; Illumina technology), or converted to cDNA for analysis by quantitative Real-Time PCR (qRT-PCR) as described earlier^{11,26}. Post-sequencing processing and gene expression analysis was done essentially as reported elsewhere¹¹. Primers and probes for qRT-PCR are listed in Table S1 and in¹¹. The RNA-Seq reads were mapped to the reference genome *Mmar*^{CCUG} with the aligner Bowtie2⁸¹. Subsequently, the read counts and differential mRNA levels were calculated using HTSeq (v0.6.0)⁸² and DESEQ2⁸³ packages, respectively.

For the sigma factors only, RNA-Seq data for one replicate of the stress and growth samples and qRT-PCR data for the heat, cold, and microaerobic conditions time course experiment was published previously¹¹ (but see⁴⁷ where we reported sigma factor transcript levels based on two replicates, and transcriptomic datasets used in³³). These data were incorporated in the analyses. Also, we previously published RNA-Seq data from one of

the replicates of the stressosome pathway proteins in exponential and stationary phases²⁶. For details and statistical analyses, see Supplementary methods.

RNA stability measurement

RNA-Seq was used to determine the half-lives (mRNA stability) for all σ and anti- σ factor mRNAs in *Mmar*^{CCUG}. Compared to Northern blotting, which has been the common way to estimate RNA half-lives in the past, RNA-Seq offers improved sensitivity and superior throughput. RNA-Seq, like Northern blotting, gives information on co-transcription when the paired end read method is used. One can detect that two genes are co-transcribed when the paired end reads align with one neighboring gene because both reads originate from the same cDNA.

Mmar^{CCUG} pDEAM5 cultures were grown at 30°C with rotation (100 rpm) in liquid Middlebrook 7H9 medium including glycerol, Tween 80, Middlebrook OADC supplement, and 100 µg/ml Hygromycin B as previously described¹¹. At OD_{600nm} ≈ 0.5, rifampicin (500 µg/ml final concentration) was added, and immediately prior the addition of rifampicin 2 ml aliquot (zero sample) of the culture was snap-frozen in liquid nitrogen. After addition of rifampicin, aliquots were withdrawn at 0.5, 1, 3, 6, and 12 min and stored at -80°C until further processing. For RNA-Seq analysis, the samples were put on ice until almost completely thawed and the cells were pelleted at +4°C by centrifugation at top speed in a table-top centrifuge. The supernatant was removed and RNA was extracted as described below.

For qRT-PCR analysis, the samples were spiked with cell pellets from 1 ml aliquots of a single *E. coli* MG1655 culture before extracting and processing the RNA for qRT-PCR as described below. Since the spike-in samples come from an external source containing fixed amounts of cells (RNA), which is added to each experimental sample, enables the determination of the "absolute" half-lives for the RNAs. The zero sample was used as the calibrator sample and the standard curve method for quantification was employed. The *E. coli* 16S rRNA was used as an internal standard (for primers and probe, see Supplementary Table S1). The half-lives were measured for *Mmar* and *M. smegmatis* (*Msmeg*) *sigA*, *sigB*, and *rshA* mRNAs from three independent experiments (two for *Msmeg*) and the average half-lives were calculated.

The RNA-Seq reads were initially checked for quality and then mapped using the *Mmar*^{CCUG} complete genome and Bowtie2⁸¹ with default parameters. The transcript abundance was calculated using the "htseq-count" tool of the HTSseq (v0.6.0) package⁸². The samples were normalized by the count of the *sigA* transcript for each time point and further calibrated by taking a ratio for each gene with the experimentally calculated half-life of the *sigA* transcript (from qRT-PCR; see above) at the corresponding time points.

Declarations

Supplementary Information

Supplementary Methods and Data are available online.

Competing interests

The authors declare no competing interests.

Author Contribution

BMFP and LAK conceived the study. LZ, BMFP and LAK wrote the manuscript. LZ, BMFP and MR conducted experiments. SD and PRKB performed the bioinformatics and processed the RNA-Seq data. LZ, BMFP, MR, PRKB, SD, SS and LAK analyzed the results. All authors reviewed the manuscript.

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Data Availability

The RNA Seq data generated and used in this study are available in the NCBI BioProject database under accessions PRJNA1468953 and PRJNA1471559; reviewer access is provided via the following links: <https://dataview.ncbi.nlm.nih.gov/object/PRJNA1468953?reviewer=dqq7i5dqfdu96p2mlm23to5tl0> and <https://dataview.ncbi.nlm.nih.gov/object/PRJNA1471559?reviewer=27s3b7qh0dke35d7plhjtbbfj>. We also used RNA-Seq data from a previous study available at NCBI BioProject PRJNA1423637. Additional information is available from the corresponding author upon reasonable request.

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Figures

Fig. 1

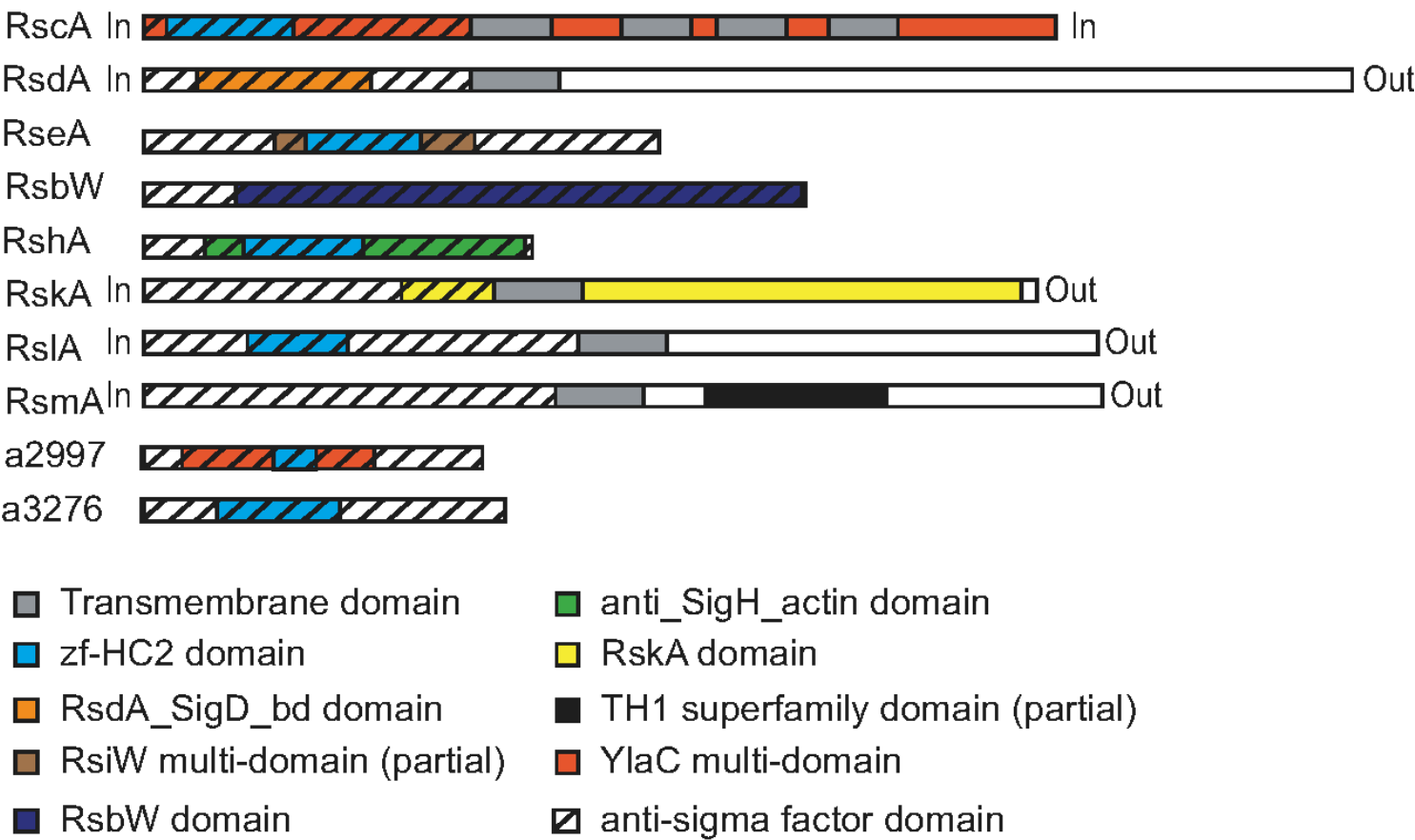


Figure 1

Domain organization and membrane topology of anti-σ factors

The predicted domain organization and membrane topology of the known and putative anti-σ factors of *Mmar* is shown (see the main text for details). "In" and "out" indicate whether the proteins N- and C-termini are on the

cytoplasmic (In) or extracellular (Out) side of the membrane. In case of multiple hits, only the domains with the lowest e-value are shown together with all detected zf-HC2 and transmembrane domains. The anti- σ factor domain (the domain interacting with the σ factor) is marked with diagonal black lines overlapping the specific domain organization. For a complete list of the detected domains and their location, and for the best hit e-values in *Mmar* and *Mtb*, see supplementary Table S2.

Fig 2

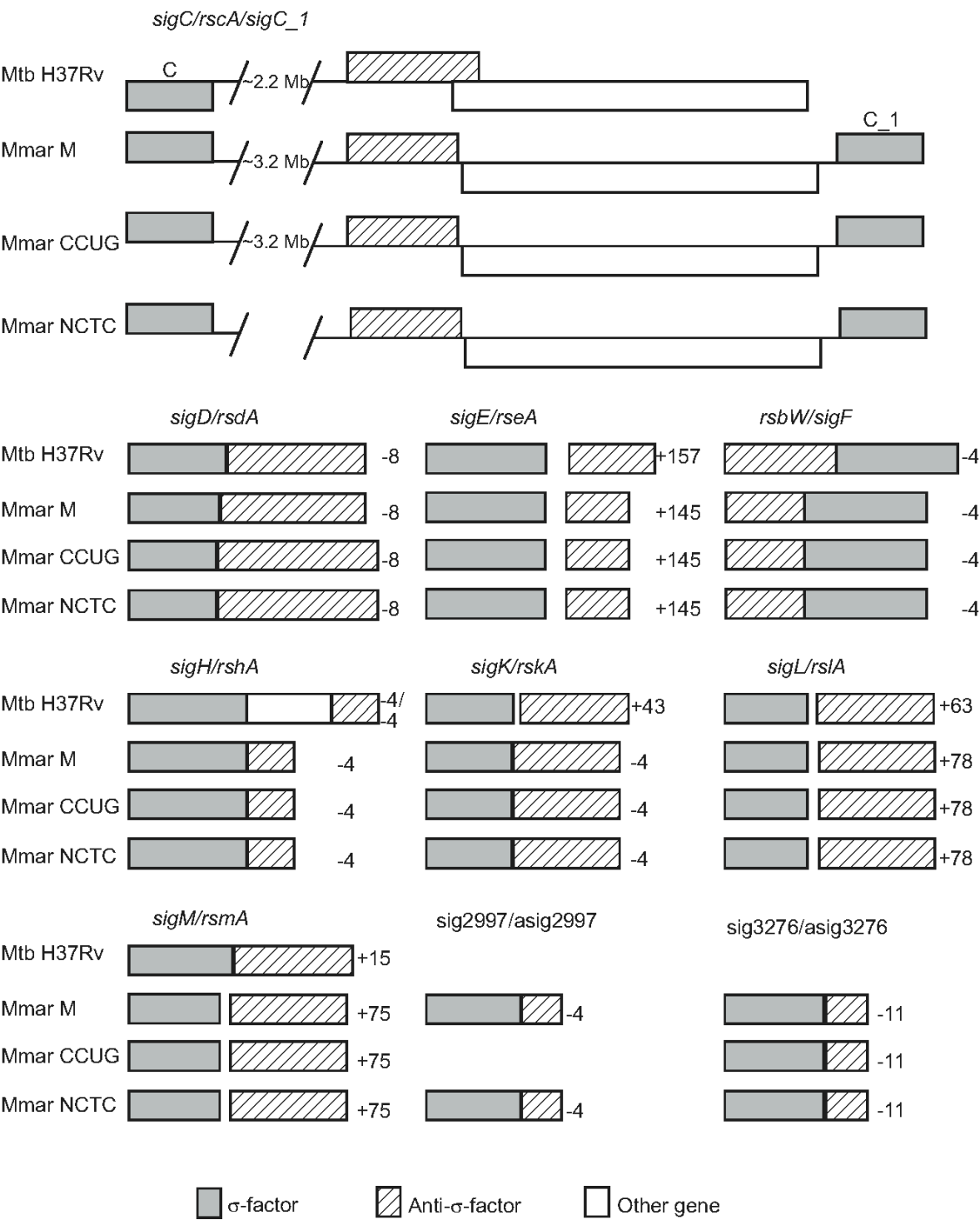


Figure 2

Gene synteny of σ factor/anti- σ factor pairs

Gene synteny for the σ factor and anti- σ factor genes of *Mtb* H37Rv and three *Mmar* strains *Mmar*^M, *Mmar*^{CCUG}, and *Mmar*^{NCTC} as indicated. The σ factor and anti- σ factor coding genes are marked by colored boxes as defined in the figure. The sizes of the boxes and the distances between them are proportional to their lengths and separation, respectively. For *sigC/rscA/sigC_1*, the box below the connecting line indicates that it is encoded in the opposite orientation. Numbers to the right of the boxes indicate the amount of overlap (negative numbers) or separation (positive numbers) between the σ factor and anti- σ factor coding genes. Empty spaces in the synteny for *sig2997/asig2997* and *sig3276/asig3276* mark genes that are absent in the respective genomes. The missing number in the separation of *sigC* and *rscA* in *Mmar*^{NCTC} is due to that the respective genes belong to different scaffolds in the genome assembly so that no distance can be accurately calculated.

Fig 3

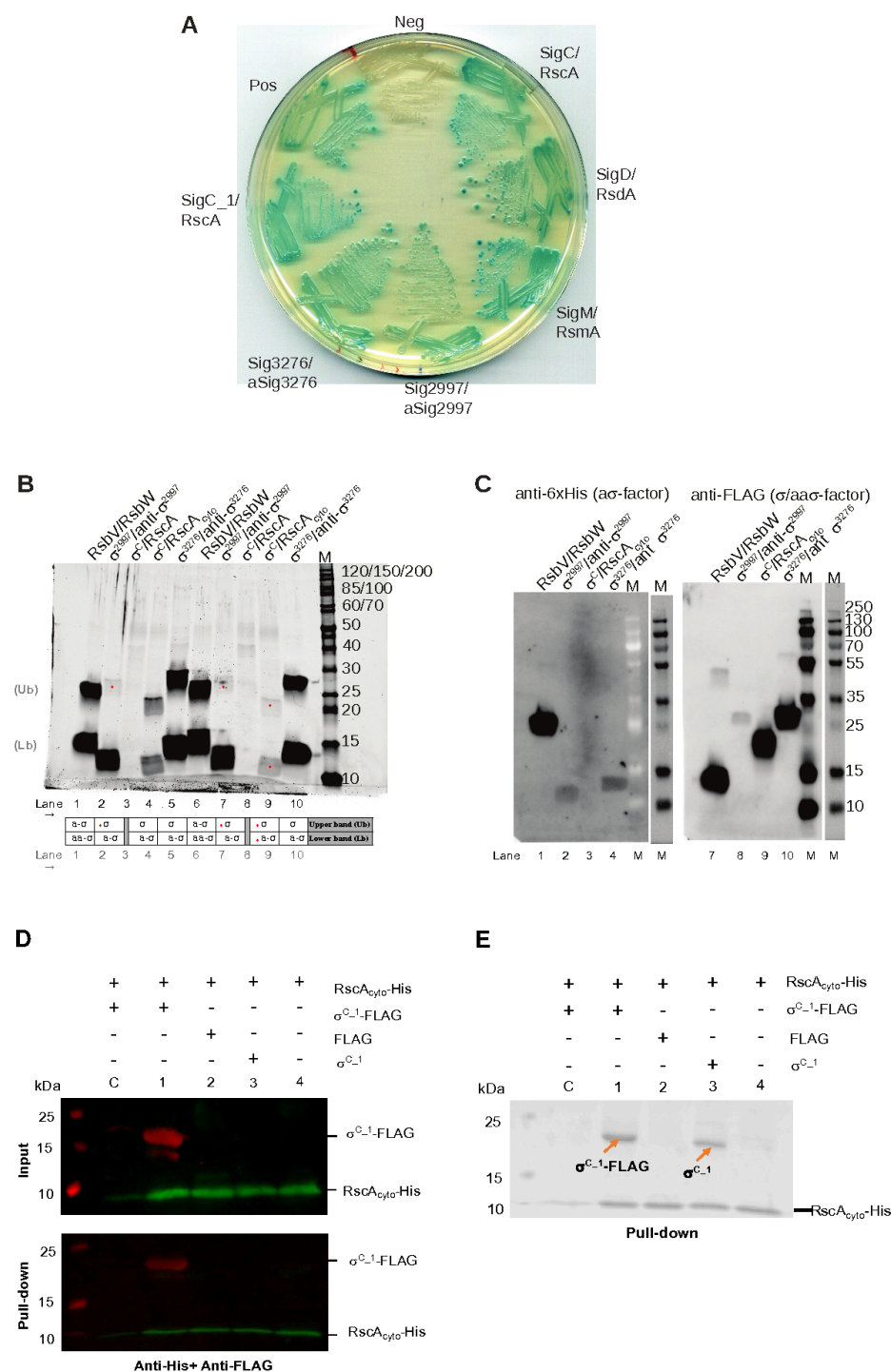


Figure 3

Positive s factor/anti- σ factor interactions

(A) Single colony streaks of *E. coli* BTH101 cells transformed with s factor and anti- σ factor pairs cloned in the bacterial two-hybrid system and grown on LB + X-gal plates supplemented with appropriate antibiotics (see Materials and Methods, and Supplementary Methods for details). Blue colonies indicate interacting proteins. As positive control (Pos), pKT25-zip and pUT18C-zip (bacterial two-hybrid system vectors with interacting leucine

zipper motifs) were used, and the empty pKNT25 and pUT18 vectors served as negative control (Neg). The full set of interactions is shown in Fig S3 and summarized in Fig S5.

(B) The 6xHis-tagged anti- σ factors were used to co-purify interacting proteins. An unstained protein size marker (M) was run alongside the samples to estimate the sizes of the detected proteins and the numbers indicate the sizes in kD. The image represents the results from duplicate samples. Lanes as indicated (see also Fig S4A-B) and the positions of the s, anti- σ (a- σ) and anti-anti- σ (aa- σ) factors are marked in the lower panel (upper, Ub, and lower bands, Lb) while * mark weak bands.

(C) The samples in (B) were electroblotted to a low fluorescence background PVDF blotting membrane and proteins were detected with antibodies detecting the 6xHis-tag (left blot) or the FLAG-tag (right blot) followed by visualization with fluorescent conjugated secondary antibodies. A pre-stained protein size marker (M) was run alongside the samples to estimate the sizes of the detected proteins and the numbers indicate the sizes in kD. The s^C/RscA sample was omitted since we could not detect any protein expression in (B). Lanes as marked, see also Fig S4C-F.

(D and E) RscA_{cyto} and s^{C-1} tagged with 6xHis- or FLAG-tags were pulled down (along with interacting partners) using anti-6xHis or anti-FLAG antibodies as indicated. C marks the control and kDa the size ladder. The experiment was conducted as described in Supplementary Methods.x

Fig. 4

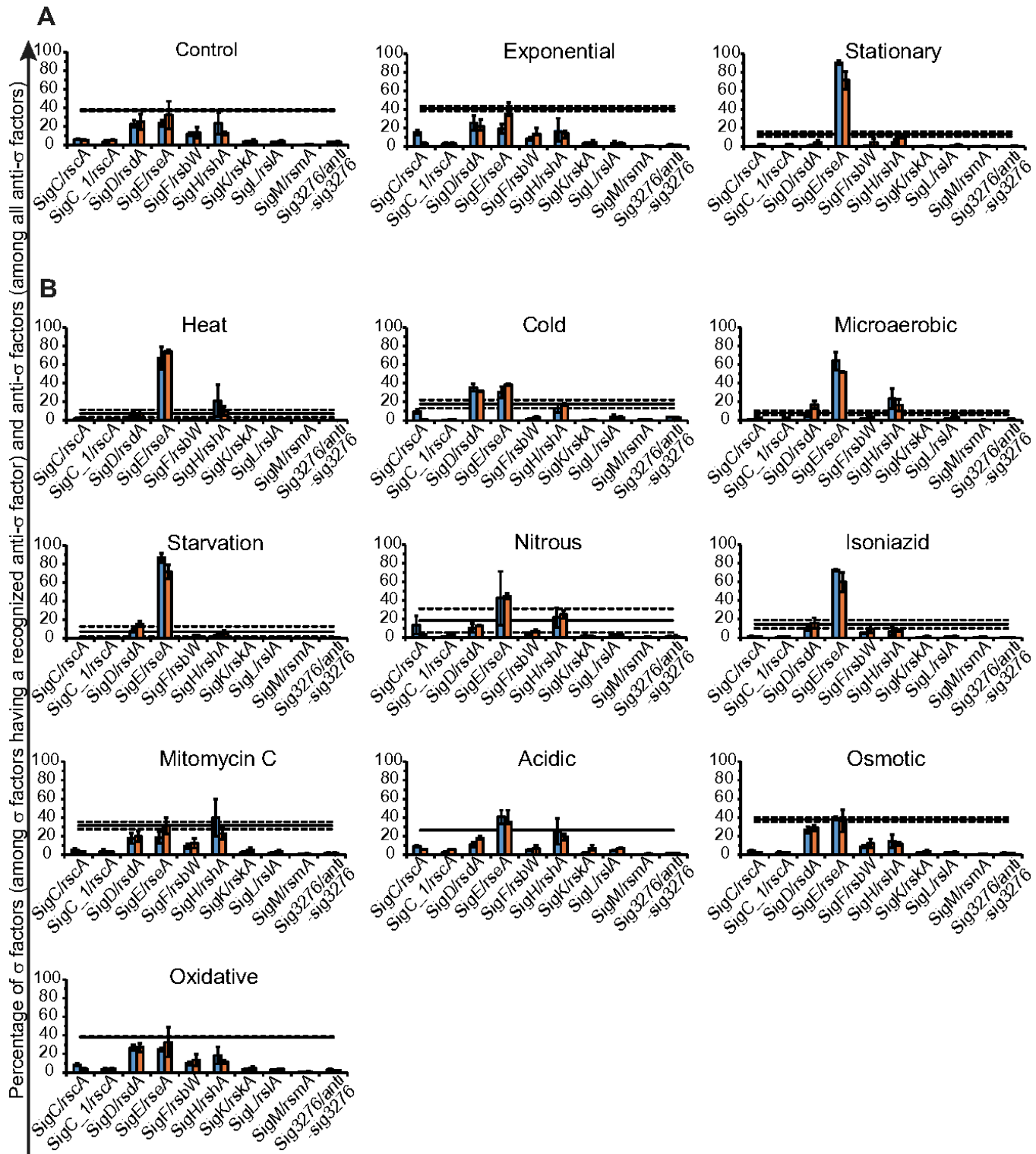


Figure 4

σ factor and anti- σ factor mRNA levels during growth and stress

The distribution (in percent) of anti- σ factors (among all anti- σ factors) and σ factors (among σ factors having a recognized anti- σ factor) was calculated and plotted for a number of growth (A) and stress (B) conditions from sequencing of total RNA pools (RNA-Seq data). σ factors are indicated in blue and anti- σ factors in red. The values are given as the average of two biological replicates with the standard errors as indicated. The horizontal

solid line shows the percentage of the housekeeping s factor σ^A among all σ factors with errors indicated by dashed lines. "Control" indicates the stress-less control sample (see the main text for details). The data relating to s factor mRNA levels taken from⁴⁷. See also¹¹.

Fig 5

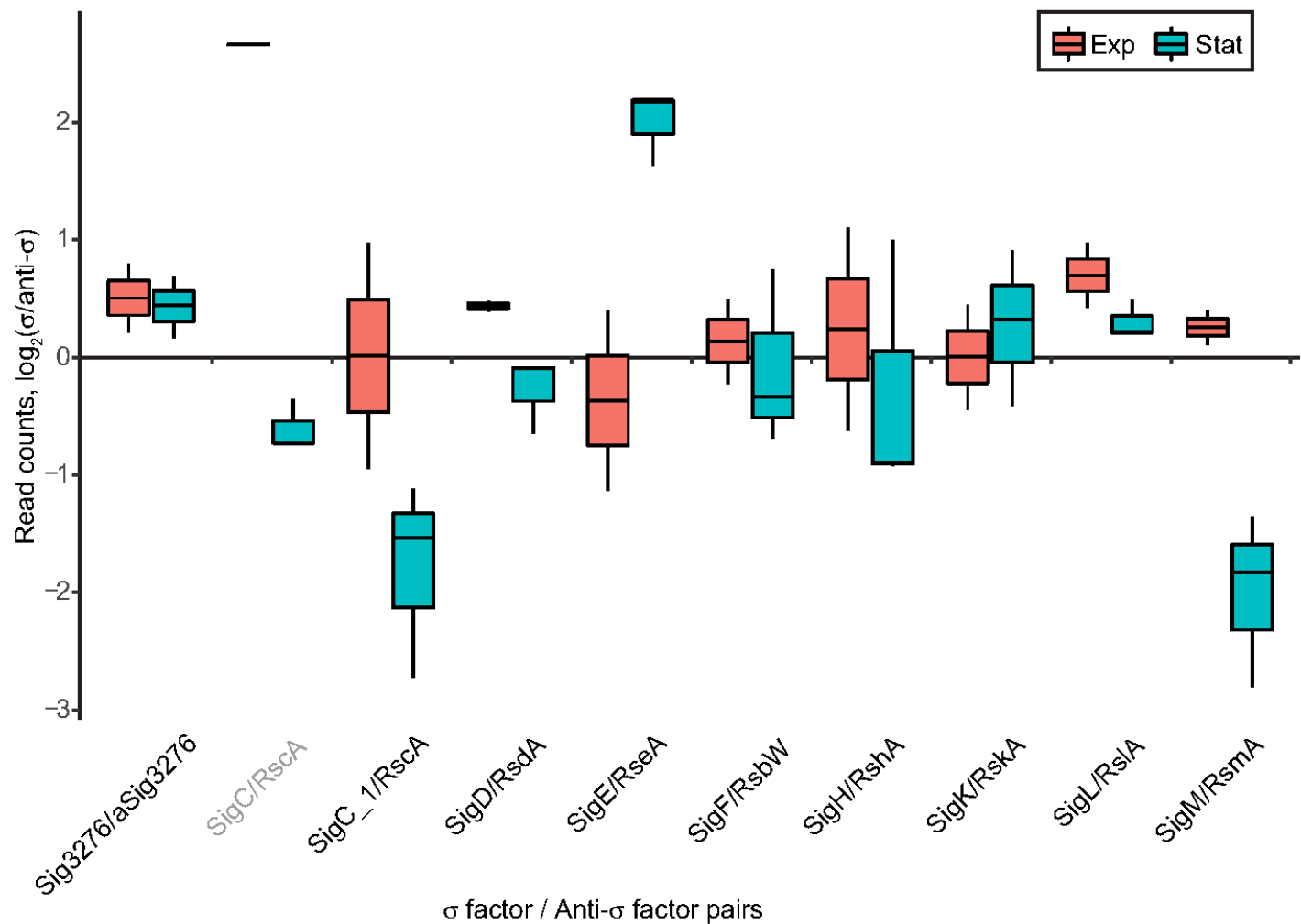


Figure 5

Ratio of absolute levels of s factor to anti-σ factor pair mRNAs in exponential and stationary phase.

Ratio of raw read counts σ factor/anti-σ factor pairs in exponential (red boxes) and stationary (turquoise) *Mmar* CCUG20998 cells. Each box plot represents the RNA-Seq mRNA transcript (raw) read counts ratio of the σ factor gene versus their cognate anti-σ factor gene plotted on log₂ scale (y-axis). Positive values indicate higher mRNA levels for σ-factor mRNA while negative values correspond to higher anti-σ-factor mRNA levels. The σ^C/anti-σ^C factor pair is coloured in grey to mark that the distance between the corresponding genes *sigC* and *rscA* in *Mmar* is ≈3.2 Mbp (see Fig 2). Red boxes represent exponential growth phase data and turquoise colored boxed stationary phase data.

Fig 6

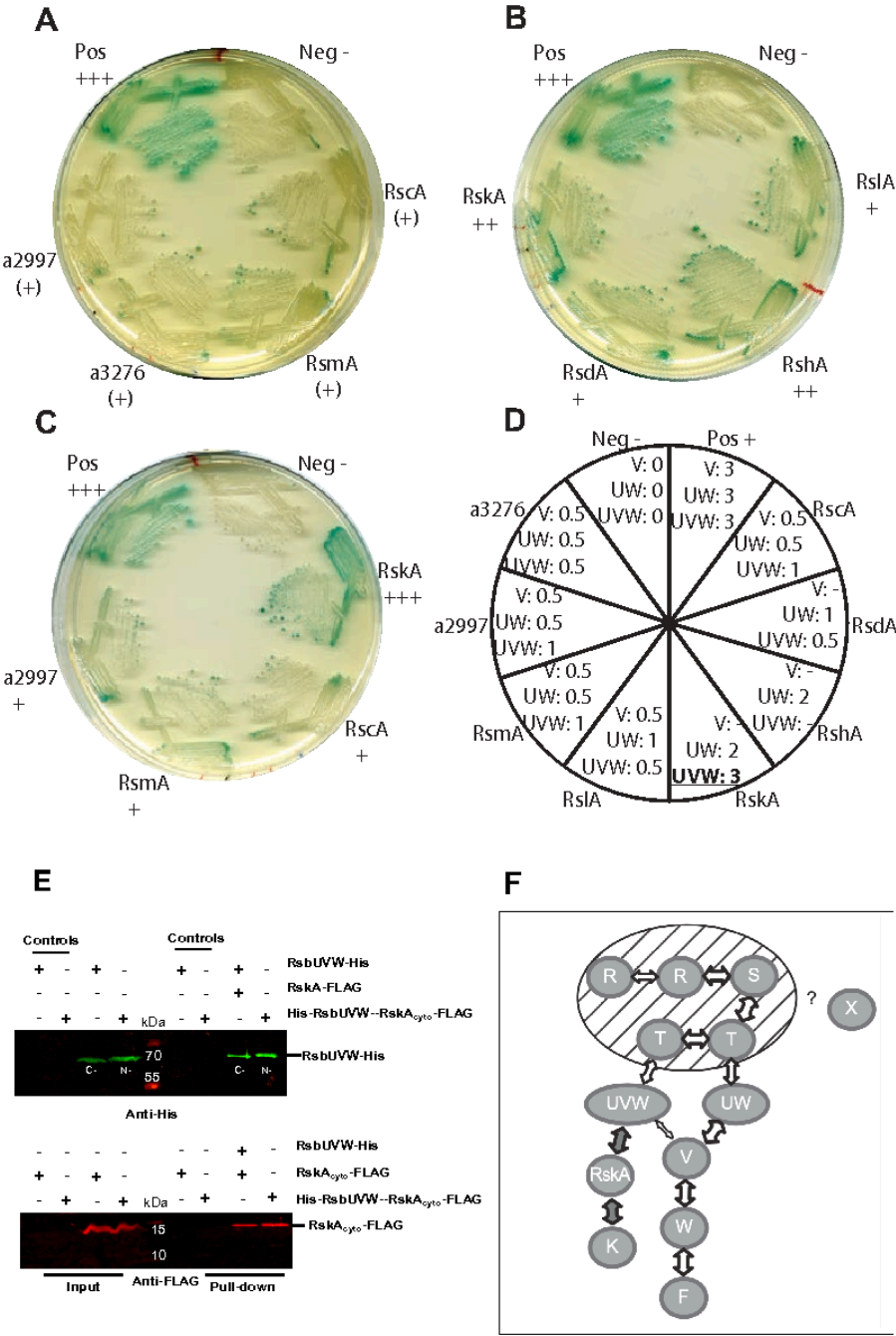


Figure 6

Anti-σ factor interactions with stressosome pathway proteins

Anti-σ factors were tested for their interaction with stressosome pathway proteins RsbV (A), RsbUW (B), and RsbUVW (C) as described in the legend to Fig 3. Weak blue color is indicated by +, intermediate blue color is indicated by ++, strong blue color is indicated by +++, very weak/questionable blue color by (+), and no blue color by -.

(D) Summary of average scores from four biological replicates where +++, ++, and + is given a score of 3, 2, and 1, respectively, and (+) a score of 0.5, and - a score of 0. Interactions with scores of at least 2.5 (indicated in bold and underlined in the figure) were considered positive.

(E) RsbUVW and RskA_{cyto} (anti- σ^K) tagged with 6xHis- or FLAG-tags were pulled down (along with interacting partners) using anti-6xHis or anti-FLAG antibodies as indicated. Controls as indicated and kDa refers to the size ladder. The experiment was conducted as described in Supplementary Methods.

(F) RsbUVW interacts with RskA (anti- σ^K) and the anti-anti- σ factor RsbV (V) interacts with the anti- σ^F factor RsbW (W). The RskA and RsbW subsequently interact with their respective cognate σ factors, σ^K and σ^F . The RsbX (X) protein is also shown for the sake of completeness. Filled gray round or oval shapes indicate proteins while double headed arrows indicate new (gray arrows) or previously demonstrated (white arrows) interactions (see also^{26,27}).

Supplementary Files

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- [Zhouetal.SUPPLEMENTARYINFORMATION.pdf](#)