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Chlamydia trachomatis type III secretion proteins regulate transcription

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Abstract:

The Scc4 protein (CT663) of the pathogenic bacterium *Chlamydia* has been described as a type III secretion (T3S) chaperone as well as an inhibitor of RNA polymerase. To examine if these roles are connected, we first examined physical interactions between *Chlamydia trachomatis* Scc4 and the T3S chaperone Scc1 and a T3S substrate CopN. In a yeast-3-hybrid assay, Scc4, Scc1 and CopN were all required to detect an interaction, which suggests that these proteins form a tri-molecular complex. We also detected interactions between any two of these three T3S proteins in a pull-down assay using only recombinant proteins. We next determined whether these interactions could affect the function of Scc4 as an inhibitor of RNA transcription. Using *E. coli* as a heterologous *in vivo* system, we demonstrated that expression of *C. trachomatis* Scc4 led to a drastic decrease in transcript levels for multiple genes. However, co-expression of Scc4 with either Scc1 or CopN, or both of these interacting proteins, alleviated Scc4-mediated inhibition of transcription. Scc4 expression also severely impaired *E. coli* growth, but this growth defect was reversed by co-expression of Scc4 either with Scc1, CopN, or both, suggesting that the inhibitory effect of Scc4 on transcription and growth can be antagonized by interactions between Scc4, Scc1 and CopN. These findings suggest that the dual functions of Scc4 may serve as a bridge to link T3S and the regulation of gene expression in *Chlamydia*.

Importance:

This study investigates a novel mechanism for regulating gene expression in the pathogenic bacterium *Chlamydia*. The *Chlamydia* type III secretion (T3S) chaperone Scc4 has been shown

to inhibit transcription by RNA polymerase. This study describes physical interactions between Scc4 and the T3S proteins Scc1 and CopN. Furthermore *Chlamydia* Scc1 and CopN antagonized the inhibitory effects of Scc4 on transcription and growth in a heterologous *E. coli* system. These results provide evidence that transcription in *Chlamydia* can be regulated by the T3S system through interactions between T3S proteins.

Introduction:

Chlamydia trachomatis is the most prevalent cause of bacterial sexually transmitted infections in the United States (3, 5). In addition, it is the most common cause of preventable blindness in the world (4). *Chlamydia* is an unusual obligate intracellular bacterium that has two distinct forms, the infectious elementary body (EB) and the non-infectious reticulate body (RB) (12). Once the EB attaches and enters a susceptible host cell, it converts into an RB that replicates by binary fission, generating hundreds of progeny within the membrane-bound chlamydial inclusion.

Similar to other pathogenic Gram-negative bacteria, *Chlamydia* utilizes a type III secretion (T3S) system to deliver effector proteins into a eukaryotic cell (9). In *Chlamydia*, T3S is important for a number of steps in the intracellular infection (13). EB entry into the host cell is mediated in part by translocation of the T3S effector protein Tarp, which recruits actin at the site of EB attachment and likely aids in internalization (6). At early and mid-stages of the developmental cycle, secretion of Inc proteins into the inclusion membrane is proposed to be important for establishing the inclusion and altering host membrane trafficking and signaling pathways (22).

Late in the developmental cycle, upon conversion of RBs to EBs, the EBs are preloaded with T3S proteins in preparation for a new round of infection (17).

T3S chaperones are known to selectively regulate and bind to T3S translocator and effector proteins (8). These chaperones have multiple functions including stabilization of T3S substrates, prevention of premature interactions between substrates through sequestration, and targeting substrates for secretion through the T3S apparatus (8). T3S chaperones are subdivided into several classes based on their substrate specificity. Class I chaperones bind to a single (class IA) or multiple (class IB) T3S effectors, class II chaperones bind translocator proteins, and class III chaperones interact with components of the T3S needle complex (15).

Scc1 and Scc4 are both class IA chlamydial chaperones that form a heterodimer (18, 20). The *Chlamydia pneumoniae* Scc1 and Scc4 heterodimer interacts with the N-terminus of CopN, which appears to have effector functions as well as serving as the putative "plug" for the T3S injectisome to prevent premature effector protein secretion (2, 7, 10, 14). *C. pneumoniae* Scc1 and Scc4 have also been shown to facilitate CopN secretion in a heterologous *Yersinia* T3S system (18). In addition to its chaperone function, *C. trachomatis* Scc4 binds RNA polymerase in region 4 of the σ subunit σ^{66} and the flap domain of the β subunit, which are both involved in -35 promoter recognition during transcription initiation (16). In an *in vitro* transcription assay, Scc4 inhibited *E. coli* RNA polymerase and a hybrid *E. coli* polymerase containing a portion of *C. trachomatis* σ^{66} (16). The significance of these relationships is not understood but they suggest that T3S and gene expression in *Chlamydia* may be linked by T3S chaperones.

In this study, we examined if the physical interactions between the *C. trachomatis* protein Scc4 and the T3S proteins Scc1 and CopN can affect its function as a transcriptional regulator. We report that Scc1 and CopN can antagonize the ability of Scc4 to block transcription and reverse Scc4-mediated growth inhibition in a heterologous *in vivo* assay. These findings provide molecular evidence of a mechanistic link between T3S and transcription in *Chlamydia*.

Materials and Methods:

***E. coli* strains and growth conditions.** *E. coli* strain XL1-Blue (Stratagene) was used for plasmid maintenance and propagation. *E. coli* strain BL21 Star (DE3) (Invitrogen) was used for expression and purification of recombinant proteins. *E. coli* strain T7 Express (New England Biolabs) was used for co-expression experiments analyzing gene expression and growth. All *E. coli* were grown in Luria-Bertani (LB) media at 37°C with appropriate antibiotics.

***C. trachomatis* culture conditions.** *C. trachomatis,* serovar D strain UW-3/Cx, obtained from the American Type Culture Collection (ATCC), was propagated in HeLa 229 cells (ATCC). HeLa 229 cells were grown in Eagle's minimal essential medium (EMEM, Life Technologies Corp.) supplemented with 5% fetal bovine serum (Atlanta Biological), 2 mM L-glutamine, and 50 µg/ml of gentamicin (Mediatech, Inc.). *Chlamydia* stocks were prepared by inoculating monolayers of HeLa 229 cells in 1-dram glass vials. After inoculation monolayers were centrifuged at room temperature for 1 h at 800 x *g* followed by the addition of EMEM containing cycloheximide (1 µg/ml). Cultures were incubated for 48 h at 37°C before being sonicated in SPG (200 mM sucrose, 20 mM sodium phosphate, 5 mM glutamate, pH7.4) and centrifuged at 500 x *g* for 10

115 min. The supernatant was removed and stored at –80°C.

116

117 **Yeast 3-hybrid assay.** *C. trachomatis copN* (full-length, N- or C-terminal truncation) was
118 cloned into the pGADT7 "prey" vector, while *C. trachomatis scc1*, *scc4*, *scc3* or *Yersinia sycE*,
119 were cloned into the two sites of the pBridge Y3H "bait" vector (Table 1). Both vectors were co-
120 transformed into the Y2H Gold yeast strain according to the manufacturer's protocol (Clontech).
121 Yeast cotransformants were plated on media lacking histidine, leucine, methionine, and
122 tryptophan, and supplemented with X-α-Gal and Aureobasidin A (Aba). A positive interaction
123 was identified by growth of blue colonies.

124

125 **Protein purification.** The 6xHis-Tag pET45b+ vector (Novagen-Merck KGaA) was used for
126 expression of recombinant 6xHis-tagged proteins. The expression vector pGEX4T-1 (GE
127 Healthcare) was employed to produce GST-fusion proteins. N-terminal 6xHis- or GST-tagged
128 recombinant chlamydial T3S proteins were purified using an affinity technique. Briefly, *E. coli*
129 BL21 was grown overnight in MagicMedia *E.coli* Expression Medium (Invitrogen), and
130 collected with centrifugation for 10 min at 4^{o}C at 5,000 x *g*. Pellets were washed and
131 resuspended in TNGS buffer, pH 7.5 (20 mM Tris-HCl, 50 mM sodium chloride, 5% glycerol,
132 0.025% N-lauroyl sarcosine). Cells were disrupted by using a French press followed by
133 centrifugation for 40 min at 4^{o}C at 30,000 x *g*. Clarified extracts and pellets were checked by
134 SDS-PAGE for the presence of recombinant proteins. Insoluble proteins found primarily in the
135 pellet were purified using a urea-denaturing protocol with subsequent re-naturing by dialysis
136 with TNGS buffer.

137

138 **Pull-down assays with purified recombinant proteins.** GST-protein fusions were immobilized
139 on glutathione-sepharose beads (100-500 ul) by incubating the purified GST-protein fusions with
140 glutathione-sepharose beads equilibrated with TEN100 (20 mM Tris, pH 7.4, 0.1 mM EDTA and
141 100 mM NaCl) at 4°C rocking for 1 h. Charged beads were washed four times with 100 volumes
142 of TEN100 to remove unbound material and resuspended in TEN100, and stored at 4°C.
143 Approximately 500 ug of purified 6xHis-fusion proteins in TEN were individually mixed with
144 the immobilized GST-fusion proteins at 4°C with nutation for 1 h. The glutathione-sepharose
145 beads were then washed four times with 100 bed volumes of TEN buffer. Interacting proteins
146 were eluted by boiling in sample buffer and were subsequently separated by SDS-PAGE and
147 visualized using Western blot analysis. The samples were separated by 10% SDS-PAGE and
148 visualized by Western blot analysis using anti-His-Scc4, anti-His-Scc1 and/or anti-His-CopN or
149 anti-GST-CopN mouse polyclonal antibodies.
150
151 **Pull-down assay with chlamydial lysate.** Pull-down experiments were performed with purified
152 Scc1 and Scc4 recombinant proteins and extracts of HeLa 229 cells infected with *C. trachomatis*
153 serovar D were prepared using a CryoMill (Retsch) and subsequently cleared by centrifugation at
154 30,000 x g for 1 h. 6xHis-tagged recombinant protein(s) alone or mixed together were bound on
155 the HisPur Cobalt resin (Thermo Scientific) previously blocked with 0.5% BSA in TNGS buffer.
156 Nonspecific interactions were removed by washing with TNGS buffer. The *Chlamydia* cleared
157 lysate (3-5 mg of total protein) in TNGS buffer was added to the recombinant protein charged
158 beads and incubated for 1 h at 4°C with agitation. Unbound proteins were washed with TNGS
159 buffer supplemented with 5 mM of imidazole. The complexes bound to the HisPur Cobalt resin
160 were denatured with the addition of SDS sample buffer with 20 mM DTT heated at 95°C for 10

min. The samples were separated by 10% SDS-PAGE and visualized by Western blot using anti-His-Scc4, -Scc1 and/or -CopN mouse polyclonal antibodies.

***E.coli* growth inhibition and sample collection.** *E.coli* T7-express strain transformed with pST44, pMT1649, pMT1652, pMT1653, pMT1654, pMT1655, pMT1667, or pMT1668 (see Table S1) were grown in LB broth with 100 ug/ml of carbenicillin. Overnight cultures were diluted to an OD_{600} of 0.05 with or without 0.1 mM IPTG induction. The OD_{600} was monitored at 0.5, 1, 2, 3 and 4 h. At 4 h aliquots of approximately $1x10^8$ bacterial cells were collected from each culture. Bacterial cells were washed twice with ice cold PBS, pelleted, and stored at -80°C. Aliquots were subsequently used to isolate DNA for genome copy quantification, RNA for qRT-PCR, and for Western blot to demonstrate protein expression.

Isolation and processing of nucleic acids. *E. coli* containing expression plasmids were grown without IPTG (uninduced) or with 0.1 mM IPTG (induced) for 4 h and aliquots collected. One aliquot for each experimental condition was processed using the DNeasy Blood & Tissue kit (Qiagen) according to the manufacturer's instructions to purify genomic DNA. DNA was eluted with 200 µl of DEPC-treated ddH_2O giving 0.5% total DNA/µl, which was then diluted to 0.01% total DNA/µl and stored at -20°C. An additional aliquot was collected in parallel under each experimental condition for isolation of RNA using the RNeasy Plus mini kit (Qiagen) according to the manufacturer's instructions, and RNA was eluted into 50 µl of DEPC-treated ddH_2O. From total RNA, 2 µl (4%) was incubated with 10 units of RQ1 RNase-free DNase (Promega) at 37°C for 60 min, after which an additional 10 units of DNase was added and incubated with RNA for another 60 min. Following DNase treatment the RNA was re-purified using the RNeasy Plus

mini kit and eluted into 200 µl of DEPC-treated ddH_2O, giving a final concentration of 0.02% of total RNA/µl.

Quantitative PCR: For analysis of DNA from *E. coli*, quantitative PCR (qPCR) using the iQ SYBR Green kit (BioRad) was performed in triplicate with primers diluted to a final concentration of 250 nM and a total reaction volume of 20 µl. As a template, 2 µl (0.02%) of total genomic DNA was added to each reaction. Reactions were carried out on a BioRad iCycler with an initial denaturation step at 95°C for 5 min followed by 40 cycles of 30 seconds at 95°C and 30 seconds at 60°C. Fluorescent detection occurred during the annealing phase and subsequently during a dissociation curve analysis to confirm amplification of a single product. The threshold cycles (Ct) were determined using the BioRad iCycler software.

Quantitative reverse-transcriptase PCR: For analysis of RNA transcript levels, qRT-PCR using the iTaq Universal SYBR Green One-Step Kit was performed in triplicate with primers diluted to a final concentration of 250 nM and a total reaction volume of 20 µl. cDNA was synthesized from 2 µl (0.04%) of DNase treated RNA for 10 min at 50°C, followed by a 5 min denaturation cycle at 95°C, and 40 cycles of 30 seconds at 95°C and 30 seconds at 60°C. Fluorescent detection and dissociation curve analysis were performed as described for qPCR. Reactions lacking reverse-transcriptase were performed in parallel for each RNA sample to confirm the absence of contaminating DNA.

qRT-PCR data analysis: To normalize transcripts to genome copy number, standard curves were generated with each gene-specific primer pair by performing qPCR on *E. coli* genomic

DNA ranging between 3 x 10^2 and 3 x 10^6 copies in each reaction. The Ct value obtained by adding 0.02% of total DNA for each sample was fit to the standard curve to determine the number of corresponding gene copies, and then multiplied by a dilution factor of 5,000 (100%/0.02%) to yield total genome copies in each *E. coli* aliquot. The Ct value obtained by adding 0.04% of total RNA for each sample using qRT-PCR was also fit to the gene-specific standard curve to determine the number of transcript copies, and then multiplied by a dilution factor of 2,500 to yield total transcript copies in each *E. coli* aliquot. To calculate relative transcripts per genome copy, the number of transcripts was divided by the number of genome copies for each paired set of aliquots. Fold change was then calculated as the relative transcripts per genome copy of induced samples divided by the relative transcripts per genome copy of uninduced samples, ((transcripts/genome induced)/(transcripts/genome uninduced)). Statistical significance of fold change differences in qRT-PCR was determined by comparing each condition to the Scc4 expressing *E. coli* strain using a Student's *t*-test.

Results:

We first investigated interactions between the *C. trachomatis* T3S proteins Scc4, Scc1, and CopN using a yeast 3-hybrid (Y3H) approach. A positive interaction with CopN required both Scc4 and Scc1 to be present, and was not detected with either Scc4 or Scc1 alone (Table 1 and Fig. S1). There was no interaction when we replaced either Scc4 or Scc1 with SycE, an Scc1 ortholog from *Yersinia*, indicating that the interactions were *Chlamydia*-specific. Using a truncated form of CopN, we mapped the interaction with Scc4 and Scc1 to the N-terminal half of

CopN. As a control, we used the C-terminus of CopN and found that it interacted with Scc3, as previously described (19), but not with Scc4 and Scc1.

To further understand the relationship between *C. trachomatis* Scc4, Scc1, and CopN, we examined two-way physical interactions between any two members of this trimolecular complex. These studies were performed with pull-down assays using recombinant purified proteins tagged at the N-terminus with either 6x-His or GST (Table 2 and Fig. S2). Interaction between Scc1 and CopN was detected in reciprocal experiments with either His-Scc1 and GST-CopN, or His-CopN with GST-Scc1. His-Scc4 interacted with GST-Scc1, and in a separate experiment with GST-CopN. However, there was no interaction detected when we used GST-Scc4 and either His-Scc1 or His-CopN, which may have been due to steric hindrance or conformational constraints caused by the GST tag. These results demonstrate that each of these three *C. trachomatis* T3S proteins can interact with one another under these *in vitro* conditions.

We then examined if we could detect interactions between Scc4, Scc1, and CopN in a pull-down assay using a lysate from *Chlamydia*-infected cells. We supplemented the lysate with recombinant His-Scc4 and His-Scc1 (rScc4 and rScc1), bound the mixture to cobalt resin and assayed for co-isolation of CopN. Native CopN was detected when both rScc4 and rScc1 were incubated with the chlamydial lysate, providing further evidence of a trimolecular complex. We were unable to detect CopN when we supplemented with either rScc4 or rScc1 alone, which suggests that the native proteins were at low abundance in the lysate.

It has been reported that *C. trachomatis* Scc4 binds the β and σ subunits of RNA polymerase, and inhibits transcription of the major form of chlamydial RNA polymerase, σ^{66} RNA polymerase, as well as *E. coli* σ^{70} RNA polymerase (16). Therefore, once we had established that these three T3S proteins physically interact, we examined whether Scc1 and CopN could alter the ability of Scc4 to inhibit transcription. Using *E. coli* as a heterologous *in vivo* transcription assay, plasmids containing one, two or all three of the chlamydial T3S genes, *scc4*, *scc1* and *copN*, were expressed. We checked the IPTG-induced expression of the chlamydial proteins by Western blot analysis and verified that Scc4 protein levels were not affected by Scc1 or CopN co-expression (Fig. S3). We then measured transcript levels of selected σ^{70}-dependent *E. coli* genes, normalizing the results to *E. coli* genome copy number to control for any differences in growth rate. Finally, we examined if particular combinations of the chlamydial T3S proteins affected transcription by comparing transcript levels with and without IPTG induction of these proteins.

Scc4 by itself caused a 22-fold reduction in transcription of a constitutively expressed gene, *recA* ($P<0.05$, Fig. 2A). When Scc4 was co-expressed with Scc1, however, there was only a 4-fold decrease in *recA* transcript levels, consistent with partial rescue of Scc4-mediated transcriptional inhibition. In contrast, when Scc4 was co-expressed with CopN, or together with Scc1 and CopN, transcript levels were restored to baseline, demonstrating that inhibition of transcription by Scc4 had been completely reversed. In a control experiment, Scc1 and CopN in the absence of Scc4 did not alter *recA* transcription indicating that these T3S proteins did not non-specifically stimulate transcription.

274 To examine if Scc1 and CopN had a general effect on the reversal of Scc4 transcriptional
275 inhibition, we performed this analysis with two additional *E. coli* genes. We chose *idnT* and *cysG*
276 because these genes are stably expressed during IPTG-induced recombinant protein expression
277 (24). Expression of Scc4 alone decreased transcription of *idnT* by 32-fold and *cysG* by 18-fold
278 ($P<0.05$, Fig. 2B-C). Similar to *recA*, Scc4-mediated inhibition was partially reversed by Scc1,
279 and transcription was restored to baseline levels by CopN, or Scc1 plus CopN. Together these
280 results demonstrate the ability of CopN and to a lesser extent Scc1 to antagonize Scc4-mediated
281 transcriptional inhibition.
282
283 The pronounced transcriptional inhibition by Scc4, and the rescue of this inhibition by Scc1 and
284 CopN, led us to investigate the effect of these three chlamydial T3S proteins on the growth of *E.*
285 *coli*. Scc4 expression inhibited *E. coli* growth, resulting in only a small increase in OD_{600} up to 4
286 h post-induction compared to the uninduced control (Fig. 3C). Scc4-mediated growth inhibition
287 was partially reversed by co-expression with Scc1 (Fig. 3D). In contrast, there was complete
288 rescue of growth inhibition when Scc4 was co-expressed with CopN (Fig 3E), or when Scc4 was
289 co-expressed together with Scc1 and CopN (Fig. 3F). In control experiments, Scc1 and CopN
290 expression in the absence of Scc4 did not alter *E. coli* growth (Fig. 3B). In contrast, Scc4 was
291 still able to inhibit *E. coli* growth when it was coexpressed with *Y. pseudotuberculosis* SycE,
292 which is a T3S chaperone similar in size and function to chlamydial Scc1 (Fig. S4). This result
293 demonstrates that reversal of Scc4 mediated growth inhibition was due to Scc1 and CopN, and
294 was not a non-specific effect from co-expression of an additional protein with Scc4. These
295 findings indicate that the growth defect produced by Scc4 was likely due to its inhibitory effect
296 on RNA polymerase and transcription. In addition, our data indicate that CopN and Scc1 are able

297 to modulate these negative effects of Scc4 on both transcription and growth, with CopN
298 demonstrating the most pronounced effect.
299
300 **Discussion:**
301
302 A distinguishing characteristic of a *Chlamydia* infection is the temporal expression of chlamydial
303 genes over the course of the intracellular developmental cycle. However, the signals that control
304 gene regulation remain largely unknown. In this study we focused on a regulator of chlamydial
305 RNA polymerase that also functions as a T3S chaperone. We showed that *C. trachomatis* Scc4
306 interacts with the T3S proteins Scc1 and CopN in Y3H and pull-down assays, which is consistent
307 with studies of their orthologs in *C. pneumoniae* (18). We then showed that these physical
308 interactions have functional significance by demonstrating that Scc1 and CopN were able to
309 antagonize the inhibitory effects of Scc4 on transcription and growth of *E. coli*. These findings
310 indicate that T3S and gene expression in *Chlamydia* could be linked by the dual functions of
311 Scc4 as a T3S chaperone and a general inhibitor of transcription.
312
313 Our data support a model in which Scc1 and CopN prevent Scc4 from binding and inhibiting
314 RNA polymerase. The experimental evidence indicates a functional role for the trimolecular
315 Scc4-Scc1-CopN complex in sequestering Scc4 and modulating its activity. Our results also
316 indicate that Scc4 can interact with CopN, and to a lesser extent Scc1, in two-way interactions.
317 However, these bimolecular interactions were detected using purified recombinant proteins in
318 our pull-down assay, and by overexpressing the chlamydial proteins in our *in vivo* transcription
319 and growth studies. Thus the physiologic relevance of the two-way interactions is unclear

because the high concentrations of the chlamydial proteins in these studies may have exaggerated the physical interactions between these proteins. Our findings of two-way and three-way interactions between Scc4, Scc1, and CopN are mostly consistent with published studies. For example, efficient secretion of *C. pneumoniae* CopN required both Scc4 and Scc1, and all three proteins were required for interactions in a pull-down assay (18). Direct interactions between *C. trachomatis* Scc4 and Scc1 in a Y2H assay have also been reported (20). However, we also discovered evidence of potential two-way interactions between CopN and either Scc1 or Scc4, which has not been previously reported.

We are aware that Scc4 could have caused an apparent decrease in transcription if there were sufficient numbers of dead bacteria to artifactually increase the genome copy number used to normalize our transcript levels. However, Scc4-expressing *E. coli* continued to divide, albeit slowly (Fig. 3), while showing very large decreases in transcription (> 18-fold inhibition for all three genes, Fig. 2). Furthermore, the ability of Scc1 and CopN to alleviate this transcriptional inhibition suggests that the inhibitory activity of Scc4 is due to a specific molecular mechanism.

T3S and gene expression are linked in a number of pathogenic Gram negative bacteria (23) (21) (1), but the mechanism in *Chlamydia* appears to have some unique features. In a common scenario in other bacteria, the T3S chaperone interacts with a transcription factor, promoting selective activation of T3S genes. For example in *Shigella flexneri*, secretion of the T3S effectors IpaB and IpaC releases the T3S chaperone IpgC, which then serves as a coactivator for the transcription factor MxiE, causing activation of T3S effectors (11). This coupling of T3S and gene expression in other bacteria is used to homeostatically regulate T3S protein levels. In

343 contrast, *Chlamydia* Scc4 appears to be a global regulator of chlamydial transcription because it
344 targets the core transcriptional machinery (16). Thus Scc4 has the potential to inhibit all genes
345 transcribed by σ^{66} RNA polymerase and not just T3S genes. Another difference is that the T3S
346 chaperone typically plays a role in transcriptional activation in other bacteria, while Scc4 has a
347 negative effect as an inhibitor of chlamydial transcription.
348
349 It is not known when Scc4 acts as a transcriptional inhibitor in the chlamydial developmental
350 cycle, and so it is difficult to predict when its activity is modulated by Scc1 and CopN. Scc4 has
351 been proposed to inhibit σ^{66} RNA polymerase at late times because Scc4 protein accumulates
352 late in the developmental cycle (16). However Scc4 is transcribed from a midcycle gene, leaving
353 unexplained how σ^{66} RNA polymerase, which is the major form of chlamydial RNA polymerase,
354 can transcribe midcycle and late genes if Scc4 is already present. Our results provide a possible
355 mechanism in which Scc4 could be bound and sequestered by Scc1 and CopN during midcycle,
356 preventing it from inhibiting RNA polymerase at that time. Disruption of the Scc4-CopN-Scc1
357 complex after late genes have been transcribed could then be a very late event in which Scc4 is
358 released to inhibit σ^{66} RNA polymerase at the end of the developmental cycle. This switch would
359 be predicted to have a global effect in downregulating chlamydial transcription, although it may
360 be selective because σ^{28} RNA polymerase, which transcribes a subset of late genes, is not
361 inhibited by Scc4 (16). This switch would also affect the availability of CopN, which is both the
362 plug for the T3S apparatus and a secreted effector, but CopN localization at late times has not
363 been determined.
364
365 In summary, we propose that the ability of Scc4 to inhibit transcription by the major chlamydial

RNA polymerase can be regulated by its interactions with the T3S proteins CopN and Scc1. This mechanism is based on the dual functions of Scc4 as a T3S chaperone and a transcriptional regulator. This functional link between T3S and transcription provides new insight into how chlamydial gene expression is regulated and could be exploited in a novel anti-chlamydial strategy targeting the temporal control of transcription during the intracellular infection.

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Figure legends:

Figure 1: Pull-down assay showing protein-protein interactions for His-tagged Scc4 and Scc1 (rScc4 and rScc1), and native CopN. Lane 1 shows CopN present in the *C. trachomatis* lysate used in the pull-down assay (input). Lanes 2-4 show proteins recovered from the pull-down when the chlamydial lysate was incubated with cobalt resin and with rScc1 alone (lane 2), rScc4 alone (lane 3), or both rScc1 and rScc4 (lane 4). Mouse polyclonal antibodies used to detect the proteins in the Western blot shown are: Lane 1, anti-His-CopN; Lanes 2-4, an antibody cocktail composed of anti-His-CopN, anti-His-Scc1 and anti-His-Scc4.

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460 Figure 2: Effect of *C. trachomatis* Scc4, Scc1, CopN, in various combinations, on the
461 transcription of three *E. coli* genes. Transcripts were measured by qRT-PCR 4 h after expression
462 of the chlamydial proteins was induced by IPTG. For each combination of chlamydial proteins,
463 transcript levels were normalized to genome copy number, and reported as a fold change
464 compared to transcript levels in uninduced cells. Scc4 expression decreased transcription relative
465 to all other experimental conditions ($P < 0.05$).

466

467 Figure 3: Effect of *C. trachomatis* Scc4, Scc1, and CopN, in various combinations, on *E. coli*
468 growth, as measured by OD_{600}. Each graph shows the growth curve in the absence (Uninduced)
469 or presence of IPTG (Induced). The x-axis shows the time after addition of 0.1 mM IPTG to the
470 induced sample. (A) empty vector plasmid, (B) Scc1 and CopN, (C) Scc4 alone, (D) Scc4 and
471 Scc1, (E) Scc4 and CopN, and (F) Scc4, Scc1, and CopN.

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Tables:

Table 1: *C. trachomatis* CopN, Scc1, and Scc4 demonstrate a trimolecular interaction in a yeast three-hybrid assay

pGADT7 (Prey)	pBridge (Bait)		
AD	**BD-site1**	**BD-site2**	**Interaction**
copN Full length	*scc1*	*scc4*	Positive
copN Full length	*scc4*	*scc1*	Positive
copN Full length	*scc1*	-	Negative
copN Full length	*scc4*	-	Negative
copN Full length	*scc1*	*sycE*[a]	Negative
copN Full length	*scc4*	*sycE*[a]	Negative
copN N-terminus	*scc4*	*scc1*	Positive
copN N-terminus	*scc4*	-	Negative
copN N-terminus	*scc1*	-	Negative
copN C-terminus	*scc4*	*scc1*	Negative
copN C-terminus	*scc3*	-	Positive

[a] *Yersinia* ortholog of *C. trachomatis scc1*

497 Table 2: Interactions detected using a pull-down assay with recombinant chlamydial Scc4, Scc1,

498 and CopN, each tagged at the N-terminus with either 6X-His or GST. NT = not tested.

499

	GST-Scc1	**GST-CopN**	**GST-Scc4**
His-Scc1	NT	Positive	Negative
His-CopN	Positive	NT	Negative
His-Scc4	Positive	Positive	NT

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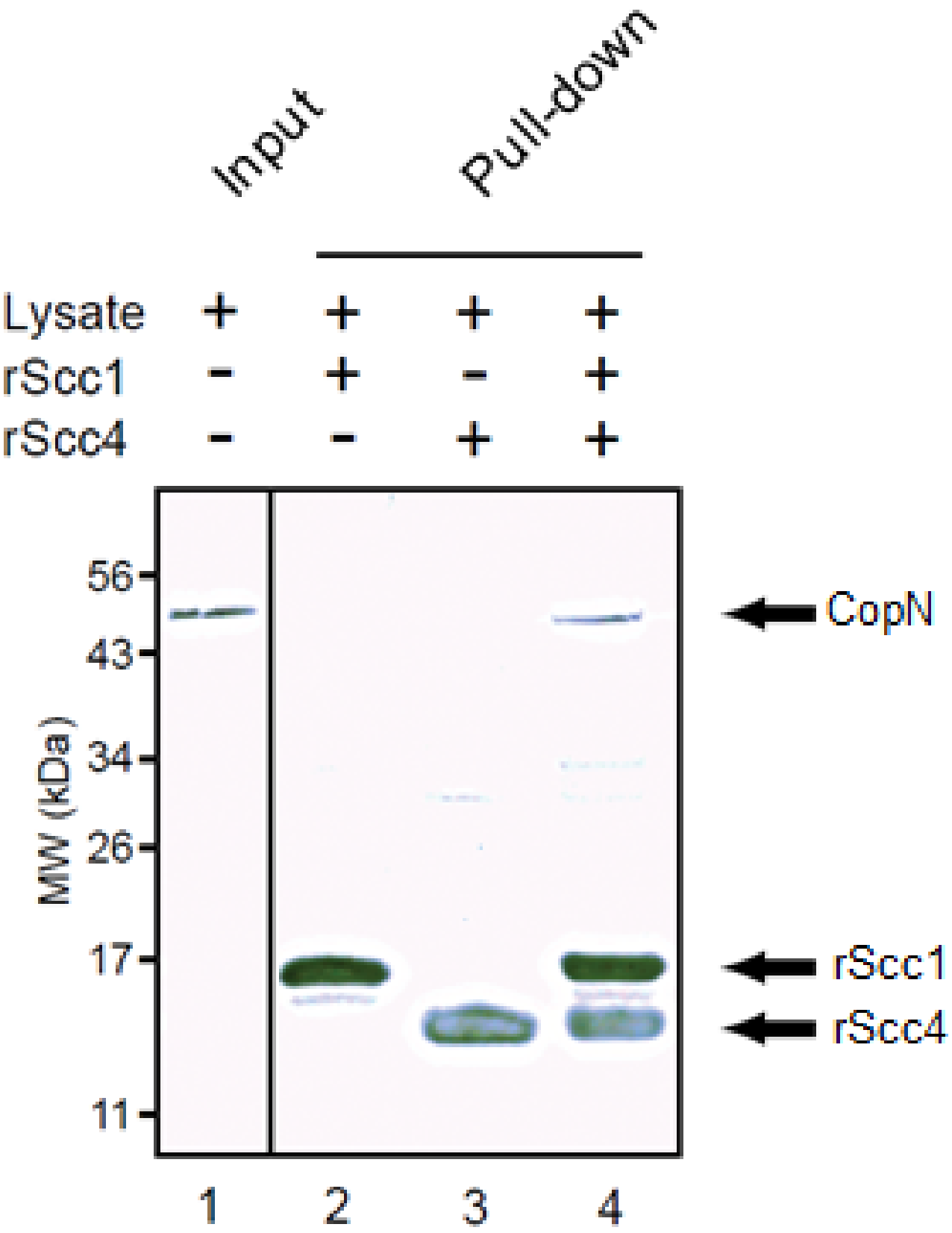

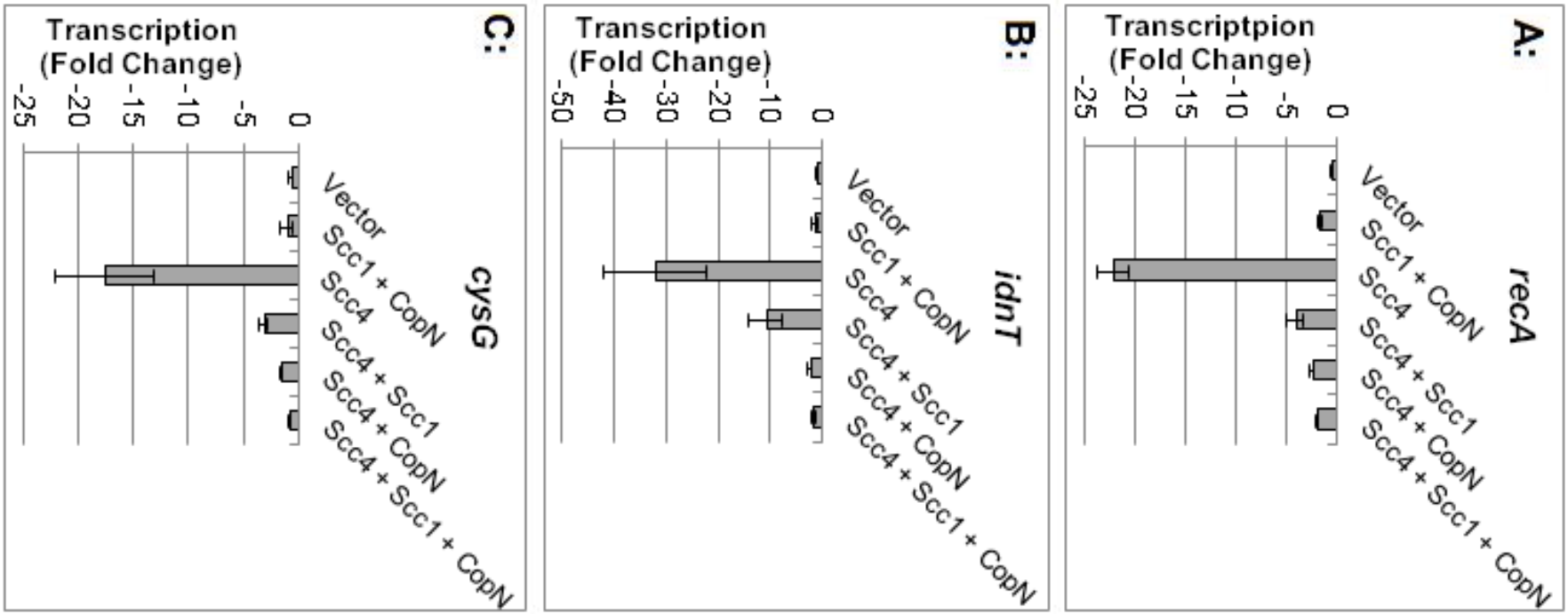
A:
recA
Transcriptpion
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-5
-10
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-20
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Vector
Scc1 + CopN
Scc4
Scc4 + Scc1
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-10
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Vector
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