Diminished nuclear RNA decay upon *Salmonella* infection upregulates antibacterial noncoding RNAs

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Abstract

Cytoplasmic mRNA degradation controls gene expression to help eliminate pathogens during infection. However, it has remained unclear whether such regulation also extends to nuclear RNA decay. Here, we show that 145 unstable nuclear RNAs, including enhancer RNAs (eRNAs) and long noncoding RNAs (lncRNAs) such as NEAT1v2, are stabilized upon *Salmonella* infection in HeLa cells. In uninfected cells, the RNA exosome, aided by the Nuclear EXosome Targeting (NEXT) complex, degrades these labile transcripts. Upon infection, the levels of the exosome/NEXT components, RRP6 and MTR4, dramatically decrease, resulting in transcript stabilization. Depletion of lncRNAs, NEAT1v2, or eRNA07573 in HeLa cells triggers increased susceptibility to *Salmonella* infection concomitant with the deregulated expression of a distinct class of immunity-related genes, indicating that the accumulation of unstable nuclear RNAs contributes to antibacterial defense. Our results highlight a fundamental role for regulated degradation of nuclear RNA in the response to pathogenic infection.

Keywords innate immune response; lncRNA; nuclear exosome; nuclear RNA degradation; *Salmonella* infection

Subject Categories Microbiology, Virology & Host Pathogen Interaction; RNA Biology

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Introduction

Numerous studies have revealed that dynamic changes in cytoplasmic mRNA abundance, through tightly regulated degradation, are crucial in the control of various biological processes (Keene, 2010; Schott & Stoecklin, 2010; Alonso, 2012). For instance, mRNAs encoding cytokines are stabilized upon the occurrence of immune stimuli through regulated binding of specialized RNA-binding proteins (RBPs) to specific sequences, such as adenylate-/uridylate-rich elements (AREs), embedded in 3' untranslated regions (3'UTRs) of these transcripts. This results in an increase in RNA abundance and the immediate production of proteins needed to fight pathogenic infection (Schott & Stoecklin, 2010). Cytoplasmic mRNA degradation is also involved in the quality control of mRNA (Lykke-Andersen & Jensen, 2015) and therefore is at the heart of post-transcriptional regulation of gene expression.

In addition to the pool of cytoplasmic mRNAs, eukaryotic genomes produce a large variety of long noncoding RNAs (lncRNAs), some of which reside in cell nuclei and regulate gene expression (Imamura & Akimitsu, 2014; Quinn & Chang, 2016). We previously identified a class of human unstable nuclear lncRNAs implicated in cell proliferation (Tani *et al.*, 2012). Other studies have identified additional unstable nuclear RNAs produced from enhancer regions, so-called enhancer RNAs (eRNAs) (Maekawa *et al.*, 2015), which may mediate the transcriptional activation of nearby genes (Tani *et al.*, 2015; Li *et al.*, 2016). Although the biological roles of the vast majority of unstable nuclear lncRNAs remain undetermined, they constitute interesting model transcripts for studying the possible impact of regulated RNA turnover on nuclear lncRNA function.

RNA degradation is primarily executed by 5'-3' and 3'-5' exonucleases. 3'-5' exonucleolytic activity in mammalian nuclei depends mainly on the multisubunit RNA exosome complex composed of nine

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core subunits, such as RRP46 (EXOSC5), and two catalytic subunits, RRP44 (DIS3) and RRP6 (EXOSC10) (Schneider & Tollervy, 2013; Januszkyk & Lima, 2014). The nuclear RNA exosome degrades a range of unstable transcripts, among which are included several lncRNAs and eRNAs (Maekawa *et al*, 2015). In addition, the RNA exosome plays a central role in the 3'-5' processing of rRNAs and 3'-extended products of snoRNAs by collaborating with the TRF4p/Air2p/Mtr4p Polyadenylation (TRAMP) complex, which resides in the nucleolus in mammals (Allmang *et al*, 1999; Fasken *et al*, 2011; Lubas *et al*, 2015; Thoms *et al*, 2015; Sudo *et al*, 2016). RNA exosome is generally guided to its substrates by RNA-binding adaptor complexes (Kilchert *et al*, 2016). Indeed, the Nuclear EXosome Targeting (NEXT) complex, consisting of the RNA helicase MTR4, the Zn-finger protein ZCCHC8, and the RNA-binding protein RBM7, is involved in the exosomal targeting of PROMoter uPstream Transcripts (PROMPTs) (Lubas *et al*, 2011), eRNAs (Meola *et al*, 2016), and the 3'-extended products from snRNAs (Lubas *et al*, 2015). In this regard, the NEXT complex contributes to the elimination of transcripts pervasively produced throughout the genome (Jensen *et al*, 2013). More recently, a nuclear RNA decay pathway, coined the Poly(A) tail eXosome Targeting (PAXT) connection, consisting of MTR4, the Zn-finger protein ZFC3H1, and the nuclear polyA binding protein PABPN1, was identified to target polyadenylated nuclear transcripts (Meola *et al*, 2016).

Here, we focus on infection by *Salmonella enterica* serovar Typhimurium (*Salmonella*), a frequently used model for investigating the mechanisms of host–bacterium interaction (Keestra-Gounder *et al*, 2015). *Salmonella* is a facultative intracellular pathogen that resides within a unique membrane-bound component following host cell invasion. Its infection mechanism depends on two type III secretion systems encoded by the *Salmonella* pathogenicity islands, SPI-1 and SPI-2, which are necessary for invasion and intracellular replication, respectively (Brawn *et al*, 2007). To limit bacterial infection, many proteins act inside host cells. However, a limited number of noncoding RNAs, including microRNAs and lncRNAs, have also been implicated in the regulation of *Salmonella* infection (Schulte *et al*, 2011; Gomez *et al*, 2013; Maudet *et al*, 2014). Here, we report on the upregulation of a subset of unstable nuclear noncoding RNAs in response to *Salmonella* infection. We find that these labile RNAs accumulate as a result of loss of nuclear exosome/NEXT components and that this accumulation contributes positively to the expression of cellular immunity-related genes and hence acts in the defense against *Salmonella* infection.

Results

Accumulation of unstable nuclear RNAs upon bacterial infection

To identify genes that are upregulated in response to bacterial infection, we conducted whole-transcriptome analysis by RNA sequencing (RNA-seq) of HeLa cells at 2, 6, and 18 h postinfection (p.i.) with *Salmonella*. This revealed that 1,210 protein-coding transcripts were upregulated with fold changes > 2 over the time course (Table EV1, Figs EV1A and EV2A). Gene Ontology (GO) terms related to immune responses were significantly enriched for these genes (Table EV2). Moreover, our data confirmed the upregulation of several mRNAs previously reported to be transiently induced upon *Salmonella* infection, such as *NFKB1*, *NFKB2*, and *RELB* (Fig EV1B) (Afonso-Grunz *et al*, 2015). Finally, of the 26,858 unstable nuclear ncRNA candidates detected in HeLa cells (Fig EV2B), 145 were upregulated with fold changes > 2 upon *Salmonella* infection with diverse induction kinetics (Fig 1A and B, Table EV3). To determine whether the upregulation of these ncRNAs could also be independently detected, we analyzed publicly available RNA-seq data from different *Salmonella*-infected cell lines (Afonso-Grunz *et al*, 2015; Westermann *et al*, 2016). We found that 15–60% of the unstable nuclear ncRNAs upregulated in *Salmonella*-infected human cells were also upregulated in different cells under different conditions (Table EV4). Human unstable nuclear ncRNAs upregulated upon *Salmonella* infection can be annotated in mouse (47 ncRNAs) and pig (34 ncRNAs) cells (Westermann *et al*, 2016). Depending on cell lines and experimental conditions, we found that 27–45% of mouse unstable nuclear ncRNAs were upregulated in response to *Salmonella* infection (Table EV4), while 15–50% of pig unstable nuclear ncRNAs were also upregulated upon *Salmonella* infection (Table EV4). This variation may reflect differences in infection conditions (e.g., time course and multiplicity of infection) or the variable cellular context (different cell types and species).

Previous studies suggested that ncRNAs can be classified into canonical lncRNAs and eRNAs (Ne *et al*, 2014). Enhancer regions may be distinguished from promoter regions by their high level of monomethylated lysine 4 residues on histone 3 (H3K4me1) and high acetylation of H3K27 (H3K27Ac) (Ernst *et al*, 2011; Dorigi *et al*, 2017). Using publicly available chromatin immunoprecipitation (ChIP)-seq data for histone modifications (ENCODE consortium), 26 of the 145 upregulated unstable nuclear ncRNAs were mapped to enhancer regions (Fig 1B lower panel, Table EV3). As eRNAs have been reported to coordinate the regulation of

Figure 1. Identification and analysis of unstable nuclear ncRNAs upregulated in response to *Salmonella* infection.

- Heat map depicting the relative expression of unstable nuclear ncRNAs (fold change > 2 comparing to noninfected condition) in HeLa cells at 2, 6 and 18 h after *Salmonella* infection (m.o.i. of 75).
- UCSC genome browser examples of NEAT1v2 from intergenic region (upper) and eRNA from enhancer region (lower) with chromatin signatures obtained from ENCODE data (<https://www.encodeproject.org/>).
- Induction of the expression of neighboring protein-coding genes of upregulated and non-upregulated eRNAs at 18 h p.i. Red and blue circles indicate induction rate of the neighboring protein-coding genes of 26 upregulated eRNAs and 100 non-upregulated eRNAs, respectively. The x-axis indicates 200-kb genomic regions (200 kb downstream and 200 kb upstream from each eRNA genes) divided into 16 bins (12.5-kb DNA region per bin). The y-axis indicates the ratio of the number of induced protein-coding genes to the number of total protein-coding genes located within each 12.5-kb DNA region, plotted as “induced genes/total genes”.
- Analysis of expression of eRNAs and the neighboring genes by ChIP–RNAPII to estimate transcriptional activation (middle panels) and by RT–qPCR analysis to estimate RNA amount (lower panels). Relative genomic positions of the analyzed genes are schematically shown in the upper panels. Arrows indicate the direction of the genes. Error bars indicate the absolute errors of two replicates.

neighboring protein-coding genes through a locus control process (Lam *et al*, 2013; Li *et al*, 2013; Melo *et al*, 2013), we investigated whether these eRNAs may be involved in the transcriptional

regulation of genes in their vicinity. To this end, we examined whether there was any correlation between the upregulation of eRNAs and their adjacently transcribed mRNAs by comparing

Figure 1.

expression changes with the distance between eRNA- and mRNA-expressing loci. This metagene analysis revealed increased expression of 70% (9/13) of protein-coding genes within a 12.5 kb genomic distance of upregulated eRNAs (Fig 1C, red line), whereas protein-coding genes adjacent to eRNAs, which were not upregulated by infection, were unaffected (Fig 1C, blue line). Interestingly, GO analysis revealed that immune-related terms were significantly enriched among the upregulated protein-coding genes (21/99) within ± 100 kb of upregulated eRNAs (Table EV5). Such eRNA-mRNA distance-dependent co-upregulation could also be detected in MTR4-depleted HeLa cells, further suggesting that the correlation indeed arose via a decrease in nuclear RNA turnover (Fig EV2C and D). Finally, we demonstrated for a subset of these cases that increased mRNA levels were in part derived from increased transcription, as determined by RNA polymerase II (RNAPII)-ChIP PCR, whereas the eRNA upregulations were not transcription-based (Fig 1D, Table EV6).

Altered half-lives underlie unstable nuclear lncRNA and eRNA upregulation

To examine the molecular basis for unstable nuclear lncRNA and eRNA upregulation, we selected four ncRNAs as model RNAs: NEAT1v2, LINC00173, eRNA07573, and eRNA10281. Of these, NEAT1v2 is a known lncRNA, which forms nuclear paraspeckles by gathering transcriptional regulators such as SFPQ and NONO (Hirose et al, 2014; Imamura et al, 2014). NEAT1v2 also participates in the transcriptional regulation of immunity-related genes in response to viral infection (Imamura et al, 2014). Living *Salmonella*, but neither heat-killed *Salmonella*, lipopolysaccharide (LPS), nor flagellin, induced the upregulation of unstable nuclear lncRNAs (Fig 2A). To analyze this further, we conducted real-time quantitative reverse-transcription PCR (RT-qPCR) assays of total RNA from cells infected with *Salmonella* mutants: SPI-1 mt (invasion) or SPI-2 mt (intercellular replication). Neither of these infections induced these unstable nuclear lncRNAs (Fig 2B). This suggests that bacterial intracellular replication triggers the increase in the tested ncRNAs.

To determine whether altered RNA stability might underlie the observed increases in ncRNA levels upon infection, we measured turnover rates of unstable nuclear ncRNAs upon *Salmonella* infection and compared these to any altered transcriptional activity. This demonstrated that infection led to the stabilization of unstable nuclear ncRNAs (Fig 2C) without any increase in RNAPII occupancy on the promoter regions of these unstable nuclear ncRNA genes (Fig 2D). In contrast, RNAPII was recruited to the promoters of the *IL8* and *SOCS3* genes, which are known to be transcriptionally activated by *Salmonella* infection (St John & Abraham, 2009; Bhavsar

et al, 2013). We conclude that unstable nuclear RNAs are accumulated due to stabilization upon *Salmonella* infection.

Factors involved in the degradation of unstable nuclear ncRNAs in uninfected cells

Several labile nuclear RNAs are degraded by the 3'-5' exonucleolytic RNA exosome (Schneider & Tollervay, 2013; Januszzyk & Lima, 2014). We therefore examined whether this is also the case for unstable nuclear ncRNAs in uninfected cells. Indeed, knockdown of the exosome core subunit RRP46 increased the abundance of unstable nuclear ncRNAs, whereas depletion of XRN2, a major nuclear 5'-3' exonuclease, had only a marginal effect (Fig 3A and B). The nucleoplasm of HeLa cells contains at least two targeting complexes associated with the nuclear RNA exosome: the NEXT complex and the PAXT connection. Moreover, the exosome co-factor hTRAMP is primarily localized to the nucleolus. Knockdown of hTRAMP components and PAXT components did not significantly affect the levels of unstable nuclear ncRNAs (Fig 3C and D). In contrast, the levels of unstable nuclear ncRNAs were increased by depletion of both the MTR4 and the ZCCHC8, components of the NEXT complex (Fig 3E). Some unstable nuclear ncRNAs were also affected by the depletion of RBM7, a component of the NEXT complex. Our data thus suggest that unstable nuclear ncRNAs are targeted for exosomal degradation mainly by the NEXT complex.

MTR4 and RRP6 are lost upon *Salmonella* infection

To search for a potential mechanism by which *Salmonella* infection might stabilize unstable nuclear ncRNAs, we used Western blotting analysis to monitor levels of different nuclear RNA degradation factors in infected cells. Interestingly, we observed that MTR4 and RRP6 were specifically lost upon infection (Fig 4A). MTR4 levels began to decrease 6 h p.i. and were almost undetectable at 18 h p.i., at which point RRP6 protein was also undetectable. To understand whether the loss of MTR4 and RRP6 could also be detected in other cell types, we analyzed several human and mouse cell lines infected with *Salmonella*. This revealed the loss of MTR4 and RRP6 in examined cell lines upon *Salmonella* infection (Fig EV3A). Consistent with this probably being causal of the upregulation of unstable nuclear ncRNAs, levels of these transcripts increased dramatically at 18 h p.i. (Figs 1A and 4B). To confirm the loss of exosome defect, we detected the upregulation of unstable nuclear ncRNAs in MTR4-depleted cells (Table EV7). To further confirm MTR4-mediated degradation of unstable nuclear ncRNAs, we determined the degradation kinetics of NEAT1v2 in MTR4-depleted or control cells. This confirmed that MTR4 depletion

Figure 2. Analysis of unstable nuclear ncRNA that accumulates upon *Salmonella* infection.

- A Expression levels of unstable nuclear ncRNAs in HeLa cells infected with *Salmonella* (m.o.i. of 75, 18 h p.i.), treated with heat-killed *Salmonella* (m.o.i. of 75), 1 μ g/ml LPS, or flagella stimuli were quantified by RT-qPCR at 18 h post-treatment. GAPDH mRNA levels were used for normalization. The dashed x-axis represents a twofold line.
- B Expression levels of unstable nuclear ncRNAs in HeLa cells infected with *Salmonella* wild type or mutants defective in SPI-1 (CS4022; *prgI*-depleted mutant) and SPI-2 (CS4019; *ssaG*-depleted mutant) were quantified by RT-qPCR 18 h postinfection. GAPDH mRNA levels were used for normalization.
- C Degradations of unstable nuclear ncRNAs were determined by BRIC (NEAT1v2) or actinomycin D chase (LINC00173, eRNA07573, and eRNA10281) followed by RT-qPCR. Colored and white circles indicate RNA levels in cells with or without *Salmonella* infection, respectively. Relative quantitative values at time 0 h were set to 100%.
- D HeLa cells with (color bars) and without (white bars) *Salmonella* infection at 18 h p.i. were subjected to ChIP-RNAPII of the promoter region of the indicated gene loci. The dashed x-axis represents a twofold line.

Data information: Values represent the mean $\pm$ SD ($n = 3$, $*P < 0.01$, Student's *t*-test).

Figure 2.

Figure 3. Involvement of the NEXT complex in the degradation of unstable nuclear ncRNAs.

A–E Level changes of the indicated unstable nuclear ncRNAs in HeLa cells transfected with the indicated siRNAs, RRP46 (A), XRN2 (B), TRAMP complex components (C), PAXT connection component (D), and NEXT complex components (E). Two independent siRNAs were examined for each target. Values represent the mean $\pm$ SD ($n = 3$, Student's *t*-test, $*P < 0.01$). Dashed lines indicate the values of twofold upregulation.

Figure 4. Loss of MTR4 and RRP6 in response to *Salmonella* infection.

A Levels of indicated proteins analyzed by Western blotting at the indicated time points after *Salmonella* infection.

B Induction kinetics of *NEAT1v2* and *eRNA07573* upregulation in response to *Salmonella* infection (m.o.i. of 75). Values represent the mean $\pm$ SD ($n = 3$, Student's *t*-test, $*P < 0.01$).

C Western blotting analysis of 5–30% glycerol gradient fractions of HeLa cell extract with or without *Salmonella* infection (m.o.i. of 75, 18 h p.i.) using the indicated antibodies.

D Western blotting analysis in HeLa cells treated with cycloheximide (CHX).

Source data are available online for this figure.

stabilizes unstable nuclear ncRNAs (Fig EV4A). Knockdown of MTR4 did not affect the expression level of RRP6, and *vice versa* (Fig EV4B), suggesting that the loss of these factors is independently executed.

To characterize the properties of nuclear RNA decay machinery during *Salmonella* infection, we ran sedimentation profiles of extracts from infected and uninfected cells on 5–30% glycerol gradients. NEXT complex components, ZCCHC8 and RBM7, were redistributed from the middle and heavy fractions (Fr. 10–22 under no infection conditions in Fig 4C) to lighter fractions (Fr. 5–14 under infection conditions in Fig 4C) at 18 h p.i. This indicates that the NEXT complex is disrupted due to the loss of MTR4 expression and would therefore result in the accumulation of unstable nuclear ncRNAs.

Because both MTR4 and RRP6 are stable proteins in naïve cells, as measured by a time-course assay harvesting samples subsequent to translation inhibition (Fig 4D), and their degradation appears to be specifically accelerated in response to *Salmonella* infection, presumably by enhanced proteolysis, the loss of MTR4 and RRP6 is an active response upon infection.

To further examine any dysregulation of MTR4 and RRP6 activities upon *Salmonella* infection, we examined rRNA processing by northern blotting analysis (Fig EV4C). In infected cells, a marked reduction in total amounts of rRNA intermediates was observed (Fig EV4D–F). Despite the relative accumulation of 32S rRNA intermediates, levels of 12S rRNA intermediates were not decreased (Fig EV4F). This is probably due to a delayed conversion of 12S into 5.8S. These results suggest that *Salmonella* infection may affect the RNA exosome-mediated rRNA processing, but not at all to the same degree as when MTR4 levels are decreased by siRNA-mediated depletion. This may be because *Salmonella* infection likely triggers more adverse effects on rRNA processing. In addition, the different durations of MTR4 depletion between siRNA-mediated MTR4 knockdown (72 h) and *Salmonella* infection (18 h) may cause the observed different alterations of rRNA processing. The mild effect on RNA exosome-mediated rRNA processing upon *Salmonella* infection also appears to be reflected by the relatively few ncRNAs that are stabilized compared with those under MTR4 knockdown conditions.

Involvement of unstable nuclear ncRNAs in the host immune response

We next examined a possible phenotype as a result of the accumulation of *NEAT1v2* upon *Salmonella* infection. *NEAT1v2* accumulation

correlated with excessive paraspeckle formation (Fig EV5A and B). Since *NEAT1* accumulation was previously shown to modulate immunity-related genes (Imamura et al, 2014), we further explored a possible role of *NEAT1v2* in host cell defense by examining the viability of *NEAT1v2* CRISPR-mediated knockout (KO) cells under conditions of *Salmonella* infection (Fig 5A). The viability of *NEAT1v2* KO cells was reduced compared with that of WT cells after *Salmonella* infection (Fig 5B). Accordingly, *Salmonella* grew well in *NEAT1v2* KO cells (Fig 5C), whereas its invasion rate into KO and WT cells was comparable (Fig 5D), suggesting that *NEAT1v2* modulates the antibacterial response after entry of the pathogen. Microarray analysis revealed 126 genes that were induced in WT but not in *NEAT1v2* KO cells upon *Salmonella* infection (Table EV8). Consistent with an antibacterial role of *NEAT1v2*, among these genes was an abundance of those involved in immune responses, such as *TNFSF9*, *CCL2*, and *CSF1* (Fig 5E, Table EV9).

Next, we examined the putative role of eRNAs in helping cells to resist *Salmonella* infection. CRISPR-manipulated KO cells of *eRNA07573*, one of the most highly stabilized eRNAs, were highly susceptible to *Salmonella* (Fig 5F and G), which grew well in these cells (Fig 5H), despite displaying similar invasion efficiencies as in WT cells (Fig 5I). Here, our microarray analysis identified 196 genes that were induced in WT but not in KO cells in response to infection (Table EV10). Finally, we investigated expression of the *SLC2A3* and *SLC2A14* genes, adjacently positioned to the *eRNA07573* locus, in *eRNA07573* KO cells. *SLC2A3* and *SLC2A14* encode glucose transporters, and cytoplasmic glucose concentration significantly affects the growth of *Salmonella* in host cells (Eisenreich et al, 2010). As shown in Fig 1D, both *SLC2A3* and *SLC2A14* were transcriptionally activated in response to *Salmonella* infection in WT cells (Figs 1D and 5J). However, this induction was significantly decreased in *eRNA07573* KO cells (Fig 5J). Since transcriptional activation of the *eRNA07573* locus was not involved in the induction of *SLC2A3* and *SLC2A14* gene expression (Fig 1D), these findings suggest that the accumulation of *eRNA07573* by RNA stabilization, rather than by transcriptional activation, affects the transcription of several genes, including near neighbors, in response to *Salmonella* infection. Overall, our data imply a similar role, namely, antibacterial response through transcriptional regulation of immunity-related genes, for at least some nuclear ncRNAs that accumulate upon *Salmonella* infection.

Figure 5. Antibacterial function of unstable nuclear ncRNAs.

- A Schematic showing the generation of *NEAT1v2* KO cells using CRISPR/Cas9. The expression levels of *NEAT1v2* in HeLa cells (white bar), in *NEAT1v2* KO-1 cells (light green bar) and in *NEAT1v2* KO-2 cells (dark green bar) as determined by RT-qPCR.
- B Survival rates of HeLa and *NEAT1v2* KO cells infected with *Salmonella* (m.o.i. of 75, 18 h p.i.).
- C Numbers of viable *Salmonella* in indicated cells after infection with *Salmonella* (m.o.i. of 75, 18 h p.i.).
- D Initial invasion number of *Salmonella* in the indicated cells immediately after infection (m.o.i. of 75, 30 min p.i.).
- E Relative expression of indicated genes involved in immune responses in infected HeLa and *NEAT1v2* KO cells compared with that in uninfected cells, as determined by RT-qPCR.
- F Schematic figure of the generation of *eRNA07573* KO cells. The expression levels of *eRNA07573* in HeLa cells (white bar), KO-1 cells (light blue bar) and KO-2 cells (dark blue bar) were determined by RT-qPCR.
- G Survival rates of HeLa and *eRNA07573* KO cells infected with *Salmonella* (m.o.i. of 75, 18 h p.i.).
- H Numbers of viable *Salmonella* in the indicated infected cells (m.o.i. of 75, 18 h p.i.).
- I Initial invasion number of *Salmonella* in the indicated cells (m.o.i. of 75, 30 min p.i.).
- J Expression of the indicated genes in HeLa and KO cells with and without *Salmonella* infection (m.o.i. of 75, 18 h p.i.).

Data information: Values represent mean $\pm$ SD ($n = 3$, * $P < 0.01$, Student's *t*-test).

Figure 5.

No Infection

Figure 6. Model of unstable nuclear ncRNA-mediated response to *Salmonella* infection.

In noninfected conditions, unstable nuclear ncRNAs are degraded by the NEXT-RNA exosome pathway. Upon infection, loss of MTR4 and RRP6 ensues and decreases unstable nuclear ncRNA turnover, resulting in the subsequent upregulation of the immune response through transcriptional activation of downstream target genes.

Discussion

The changing of cytoplasmic mRNA levels via the induction of degradation or stabilization plays an important role in responses to stresses such as infection (Schott & Stoecklin, 2010). However, the biological significance of nuclear ncRNA regulation mediated by changing transcript half-lives has remained unclear. In this study, we show that unstable nuclear ncRNAs, some of which are involved in the antibacterial immune response, accumulate due to their stabilization upon *Salmonella* infection. This response is probably caused by loss of the nuclear RNA decay factors, MTR4 and RRP6, which are involved in the degradation of unstable nuclear ncRNAs. To our knowledge, this is the first report showing that bacterial infection triggers the loss of nuclear RNA decay factors, resulting in the accumulation of unstable nuclear ncRNAs, which play important protective roles for host cells (Fig 6).

It was previously reported that lncRNAs are induced during immune responses associated with exposure to bacterial components or synthetic immune inducers. For example, *lincRNA-Cox2*, a transcriptional regulator of immune genes, is transcriptionally induced in mouse bone marrow-derived macrophages by Pam₃CSK₄,

a Tlr2 ligand, through the NF- κ B pathway (Carpenter *et al*, 2013). In addition, several lncRNAs and eRNAs, including *IL7b-eRNA*, were induced after LPS treatment by NF- κ B-mediated transcriptional activation in human primary monocytes and human monocytic THP-1 cells (Mao *et al*, 2015). Moreover, it was recently observed that suppression of the immune regulatory microRNAs miR-155 and let-7 results in increased expression of the key cytokines interleukin (IL)-10 and IL-6 during *Salmonella* infection (Schulte *et al*, 2011). Furthermore, epigenetic activation of the interferon- γ locus is regulated by an enhancer-like lncRNA termed NeST (nettoie *Salmonella* pas Thriller's) (Gomez *et al*, 2013), and several members of the miR-15 family are involved in inhibiting *Salmonella* infection (Maudet *et al*, 2014). Finally, dual RNA-seq revealed that the *Salmonella* small RNA PinT affects the JAK-STAT pathway in host cells (Westermann *et al*, 2016). Focusing on gene expression in host cells infected by *Salmonella*, we were able to identify a novel set of unstable nuclear ncRNAs involved in the antibacterial response in the present study. Although known lncRNAs were upregulated by immune stimuli via transcriptional induction, we identified several ncRNAs that were upregulated by alteration of their half-lives (Fig 6). Indeed, *NEAT1v2* is induced by viral infection through

transcriptional activation mediated by the TLR3 pathway, resulting in the activation of downstream genes such as interleukin-8 (Imamura *et al*, 2014). Here, we show that *NEAT1v2* can also accumulate through increased stability, which is a rare example of an lncRNA whose expression can be determined by both transcriptional and post-transcriptional regulation depending on the stimulus. Accumulated *NEAT1v2* regulates several immunity-related genes (Imamura *et al*, 2014). The sequestration of transcription factors into paraspeckles seems to be involved in the activation of downstream genes under *Salmonella* infection. Compared with viral replication in host cells, bacterial growth is generally slow. This might enable cells to accumulate RNA through stabilization, which is likely to be a slower mode for the upregulation of transcripts than transcriptional activation. At this point, it is unclear why host cells developed RNA stabilization-mediated upregulation of lncRNAs.

Recent studies have revealed that the mammalian genome produces an enormous number of eRNAs (Kim *et al*, 2010; Andersson *et al*, 2014). Although their roles remain largely unclear, research has indicated some function in the regulation of gene expression. For example, eRNAs can participate in the chromosomal looping between enhancers and promoters of target genes in transcriptional regulation (Lam *et al*, 2013; Li *et al*, 2013; Melo *et al*, 2013). Several studies have reported that eRNAs regulate their neighboring genes through the remodeling of chromosome structures (Li *et al*, 2013; Cinghu *et al*, 2017; Dorighi *et al*, 2017). Of particular interest to the present study, eRNAs have also been implicated in inflammatory responses and differentiation (Lam *et al*, 2013; Ne *et al*, 2014). Accordingly, our analyses show that protein-coding genes in the vicinity of accumulating eRNAs are transcriptionally activated (Fig 1C and D) and that many of these genes are involved in the immune response. In line with these studies, we propose that eRNA accumulation upon *Salmonella* infection activates the transcription of neighboring genes. We also observed the co-upregulation of eRNAs and their neighboring genes (these gene sets were randomly selected) upon small interfering (si)RNA-mediated MTR4 depletion (Table EV11). These results support the idea that accumulated eRNAs are involved in regulating the expression of neighboring genes. It remains to be investigated whether such transcriptional activation occurs through the formation or maintenance of chromosomal structures.

A recent study showed that RBM7, an RNA-binding component of the NEXT complex, is loaded onto short-lived exosome substrates, resulting in their elimination from mammalian nuclei (Lubas *et al*, 2015). In accordance with this, a subset of unstable nuclear ncRNAs were targeted by the NEXT-mediated RNA decay pathway. However, the expression level of some unstable nuclear ncRNAs was not markedly affected by RBM7 depletion (Fig 3E), although they still responded to the depletion of MTR4 and ZCCHC8. This suggests that either our RBM7 knockdown did not suffice to affect these unstable nuclear ncRNAs or that other distinct RNA decay pathways, utilizing MTR4 and ZCCHC8, serve to degrade some unstable nuclear ncRNAs.

NEAT1 lncRNA appears in two isoforms: *NEAT1v1* (short isoform) and *NEAT1v2* (long isoform). Although the biological function of *NEAT1v1* is unclear, *NEAT1v2* is essential for the formation of paraspeckles (Sasaki *et al*, 2009). *NEAT1v2* regulates several genes through paraspeckle formation (Hirose *et al*, 2014; Imamura *et al*, 2014). Exosome dysfunction leads to an

upregulation of *NEAT1v1* in HEK293T or 293A-TOA cells (Bresson & Conrad, 2013; Szczepinska *et al*, 2015). However, the mechanism of *NEAT1v2* degradation was unclear. In this study, we showed that *NEAT1v2* was degraded by RNA exosome-mediated pathway.

Remarkably, MTR4 and RRP6 levels drop swiftly in response to *Salmonella* infection, which we suggest is the underlying cause of upregulated unstable nuclear ncRNAs. The number of upregulated ncRNAs upon *Salmonella* infection (145 ncRNAs) is smaller than in MTR4-depleted cells (more than 2,000), likely because *Salmonella* infection induces more additional signals than MTR4 depletion and MTR4 knockdown (72 h) and *Salmonella* infection (18 h) lasted for different durations. Previous studies examining *Salmonella* infection also revealed the functional relevance of proteins that are depleted during infection. For example, it was found that the depletion of peroxiredoxin-6, isoform β -4c of integrin β -4, isoform 1 of protein lap2 (erbin-interacting protein), and stomatin, which are annotated to play a role in membrane traffic in the Golgi fraction, are all involved in increasing *Salmonella* replication (Vogels *et al*, 2011). However, the mechanism by which nuclear and Golgi proteins are lost remains unknown. Pathogenic infection activates caspase-1, a protease that regulates the processing and secretion of interleukin-1 family members (Ogura *et al*, 2006). Additionally, the proteasome is activated by infection (Kammerl & Meiners, 2016). However, our investigation into the presumed proteolysis of MTR4 and RRP6, using inhibitors of the proteasome (MG-132) and caspase-1 (V-zVAD-fmk), respectively, did not explain this response to *Salmonella* infection (data not shown). Thus, there remains a need to explore the molecular mechanism by which cells diminish MTR4 and RRP6 upon infection.

Materials and Methods

Reagents and molecular biological products

All chemicals were purchased from Wako Chemical (Osaka, Japan), unless otherwise stated.

Cell culture and transfection

HeLa TO cells (Clontech, Palo Alto, CA) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Life Technologies, Grand Island, NY) and antibiotics (Life Technologies, Grand Island, NY) at 37°C in a humidified incubator (Thermo Fisher Scientific) with 5% CO₂.

Reverse-transcription–quantitative real-time polymerase chain reaction (RT–qPCR)

Isolated RNA was reverse transcribed into cDNA using the PrimeScript RT Master Mix (Takara, Osaka, Japan). cDNA was amplified using the primer sets listed in Table EV12 by using SYBR Premix Ex Taq II (Takara) in accordance with the manufacturer's instructions. RT–qPCR analysis was performed using a Thermal Cycler Dice Real Time System (Takara). *GAPDH* mRNA was used for transcript normalization. Primers used in this study are shown in Table EV12.

Chromatin immunoprecipitation (ChIP) assay

Chromatin was cross-linked with 1% formaldehyde for 10 min at 37°C and then sonicated in lysis buffer (1% SDS, 10 mM EDTA, and 50 mM Tris-HCl pH 8.0 supplemented with protease inhibitor cocktail [Sigma-Aldrich, P8340]). After centrifugation, 10 µl of the supernatant was saved as input, and the remaining lysate was subjected to a ChIP assay using anti-RNAPII mouse monoclonal antibody (clone 8WG16, Abcam, ab817). The primers used for amplification are listed in Table EV13.

siRNA-mediated protein depletions

All siRNAs were designed using siDirect 2.0 Web server (<http://siDirect2.RNAi.jp/>) and were synthesized by Hokkaido System Science Co. Ltd. (Sapporo, Japan). siRNAs (Table EV14) were transfected into cells using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Briefly, cells treated with siRNA duplexes (final concentration 10 nM) mixed with Lipofectamine RNAiMAX were harvested 72 h after transfection.

Salmonella infection

The *S. enterica* serovar Typhimurium virulent strain used in this study was χ 3306. A total of 1×10^5 HeLa cells in each well of a 12-well plate were challenged with *Salmonella* at a multiplicity of infection (m.o.i.) of 75, unless otherwise stated. Plates were centrifuged for 5 min at $500 \times g$ to enhance and synchronize infection. Cells were incubated for 1 h at 37°C to permit phagocytosis, and free bacteria were removed by three washes with prewarmed phosphate-buffered saline (PBS). DMEM containing 10% heat-inactivated FBS and 100 µg/ml gentamicin was added, and the cells were incubated for an additional 2 h at 37°C in a humidified incubator with 5% CO₂. Cells were subsequently incubated with DMEM containing 10% heat-inactivated FBS and 10 µg/ml gentamicin at 37°C in a humidified incubator with 5% CO₂ for the indicated period.

Construction of *Salmonella* mutant strains

Strains, CS4019 (Δ ssaG) and CS4022 (Δ prgI), were constructed by λ Red and FLP-mediated recombination essentially as described by Datsenko and Wanner (2000). PCR products used to construct gene replacements were generated using template plasmid pKD4 and the primer sets;

ssaG-P1; 5'-CAGAACAACGTGCGCCGGAGTAATCGTTTTCAGGTA TATACCGGTGTAGGCTGGAGCTGCTTC-3'

ssaG-P2; 5'-CAGCAGCAACCGTCAACATCGTCGCTAATAAATTCA GATCATATGAATATCCTCCTTAG-3'

prgI-P1; 5'-CCAGGCCATTGGTATTTCCCAAGCCCACTTTAATTTAA CGGTGTAGGCTGGAGCTGCTTC-3'

prgI-P2; 5'-GGACAATAGTTGCAATCGACATAATCCACCTTATAAC TGACATATGAATATCCTCCTTAG-3'

Each 1,476-bp fragment generated was purified and introduced into strain χ 3306 carrying pKD46, encoding the λ Red recombinase, by transformation. To remove the Km cassette, pCP20, encoding FLP recombinase, was introduced into the resultant strains by transformation. The FRT insertion in *ssaG* or *prgI* was verified by PCR

amplification of chromosomal DNA with the primer sets ssaG-F, 5'-CAGCAGCCCATTTATATCG-3' and ssaJ-R, 5'-ACGATAAAGTTGCAA CACCTCGGG-3' or prgI-F, 5'-CAAGAAAGAGCTCAGGTGT-3' and prgJ-R, 5'-GCAAGGTCATTACCAGCAG-3'.

Assay for *Salmonella* growth in HeLa cells

HeLa cells infected with *Salmonella* were processed at the indicated time after inoculation by aspirating the medium, washing three times with PBS, and lysing with PBS containing 0.2% Triton X-100. Triplicate samples were plated on Luria-Bertani medium after appropriate dilution, and the number of colonies was counted after overnight incubation at 37°C.

Measurement of HeLa cell viability upon *Salmonella* infection

HeLa cells infected with *Salmonella* (m.o.i. of 50 or 75, 18 h p.i.) were mixed with an equal volume of 0.1% trypan blue (Life Technologies) and immediately observed under a microscope. The number of trypan blue-negative and trypan blue-positive cells was counted using a cytometer.

Immunoblotting

Cell lysates were prepared using 1× PBS supplemented with 1% Nonidet P-40, 1× protease inhibitor cocktails (Roche, Switzerland) and 50 µg/ml phenylmethylsulfonyl fluoride (PMSF). Lysates were resolved by 10% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membrane (Millipore) using a semi-dry blotter (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions. The PVDF membranes were incubated with the indicated primary antibodies, followed by incubation with either anti-mouse or anti-rabbit secondary antibodies conjugated to horseradish peroxidase (HRP). After addition of HRP substrate (Millipore), the chemiluminescence signal was detected with a Luminescent Image Analyzer LAS-4000 (Fujifilm). Antibodies used in this study are shown in Table EV15, and the results are shown in Fig EV5C.

RNA fluorescence *in situ* hybridization (FISH) and immunocytochemistry

FISH analysis was carried out to visualize endogenous RNAs according to a previously described protocol (Imamura et al., 2014). Briefly, DIG-labeled antisense RNA probes were detected with standard immunocytochemical procedures using anti-DIG sheep monoclonal antibody (Roche, Switzerland) and Alexa Fluor 488-conjugated anti-sheep IgG polyclonal goat antibody (Invitrogen). To visualize nuclear proteins, we used anti-SFPQ/PSF rabbit antibody (B92; Sigma, St. Louis, MO) and anti-NONO/p54 mouse monoclonal antibody (kindly donated by Dr. Fox, University of Western Australia). The secondary antibodies were Alexa Fluor 594-conjugated anti-mouse IgG polyclonal goat antibody (Invitrogen) and Alexa Fluor 647-conjugated anti-rabbit IgG polyclonal goat antibody (Invitrogen). For detection of DNA, samples were incubated with DAPI. Fluorescent images were obtained using a confocal microscope TCS SP5 with a 633/1.4 NA HC PL APO CS lens (Leica Co., Ltd., Tokyo, Japan). Acquired confocal images were pseudocolored and merged using the image analysis software LAS AF (Leica Co.,

Ltd., Tokyo, Japan). Stacks of optical sections with an axial distance of 250 nm were collected for each image. All images shown are representative of several repeated experiments. *Salmonella* infection was assessed by staining of *Salmonella* DNA with DAPI. For calculation of *NEAT1v2* intensity in paraspeckles, each optical section image was integrated to a single image and the fluorescence intensity of *NEAT1v2* foci was measured by using the image analysis software LAS AF.

Massive sequencing analysis

RNA-seq libraries were sequenced according to the standard protocol from mRNA-seq Sample Preparation (Illumina Inc., San Diego, CA). Briefly, approximately 1 µg of total RNA was used to prepare an RNA-seq library using the mRNA-seq Sample Preparation Kit (Illumina Inc.) in accordance with the manufacturer's protocol. Thirty six-bp single-end-read RNA-seq samples were generated from the Illumina HiSeq2000 sequencer in accordance with the standard protocol. Fluorescent images were processed to nucleotide sequences using Pipeline software supplied by Illumina. Reads mapping to ribosomal RNA genes were removed. Filtered sequences were mapped to the reference human genome (hg19) using Tophat (version 2.0.8) (Trapnell *et al*, 2009). FeatureCounts was used to assign reads to the gene annotation constructed in previous studies based on GENCODE v19 data and FANTOM5 data (Imamachi *et al*, 2014; Maekawa *et al*, 2015). Differential expression analysis was performed using Bioconductor package edgeR (version 3.12.1). A FDR cutoff of 0.1 was applied. To define eRNAs expressing from enhancer regions, we selected the eRNAs that overlapped with enhancer regions identified by the co-occurrence of H3K4me1, H3K4me3, and H3K27ac, obtained from the ENCODE project (<ftp://hgdownload.cse.ucsc.edu/goldenPath/hg19/encodeDCC/wgEncodeBroadHistone/wgEncodeBroadHistoneHelas3H3k04me1StdPk.broadPeak.gz>, <ftp://hgdownload.cse.ucsc.edu/goldenPath/hg19/encodeDCC/wgEncodeBroadHistone/wgEncodeBroadHistoneHelas3H3k4me3StdRawDataRep1.fastq.gz>, <ftp://hgdownload.cse.ucsc.edu/goldenPath/hg19/encodeDCC/wgEncodeBroadHistone/wgEncodeBroadHistoneHelas3H3k27acStdPk.broadPeak.gz>). We also used the RNA-seq data of *Salmonella* infection (DRA003692), MTR4 knockdown conditions (DRA003583), SAGE profiling (GSE61730) (Afonso-Grunz *et al*, 2015), and Dual RNA-seq (GSE60144) (Westermann *et al*, 2016).

DNA microarray analysis

Total cellular RNA was isolated using RNAisoPlus (Takara). Labeled cRNA was generated from 100 ng of total RNA using a Low Input Quick Amp WT Labeling Kit (Agilent Technologies, Palo Alto, CA) according to the manufacturer's instructions and hybridized to SurePrint G3 Human Gene Expression Microarray 8 × 60K v3.0. Microarray slides were scanned using the SureScan microarray system (Agilent Technologies), and scanned images were analyzed by Feature Extraction (ver. 11.5). To analyze expression data at the genetic level, the intensity of the signal values was summarized using Entrez Gene ID (normalized to the 75th percentile) by GeneSpring (ver. 13.0) (Agilent Technologies). The gene set probes were filtered by expression percentile (20.0–100.0%). Genes that were expressed at lower than the 20th percentile in all of the six conditions tested (uninfected HeLa cells, uninfected KO1 cells, uninfected

KO2 cells, infected HeLa cells, infected KO1 cells, and infected KO2 cells) were eliminated from the analyses. After excluding the gene set probes that did not have gene symbols, the remaining genes were used for further analysis. Welch's *t*-test was carried out to identify genes with altered expression upon infection. In this study, we only determined altered protein-coding genes. Hierarchical cluster analysis was performed using average linkage and Pearson correlation as a measure of similarity using R package obtained from Bioconductor (<https://www.bioconductor.org/packages/release/bioc/>).

Generation of knockout HeLa cell lines

Cell lines were established using the CRISPR/Cas9 system. Single guide RNAs (sgRNA) were designed using CRISPR direct (<http://crispr.dbcls.jp/>) and cloned into a pX330 vector (#42230) obtained from Addgene (<https://www.addgene.org/>). The sgRNA sequences were 5'-GGTGGCGCTGGTCTGTGTGGA-3' for *NEAT1v2* and 5'-CCGC GCCGAACACTACAATTCTTG-3' for *eRNA07573*. Targeting constructs for gene knockout were designed to insert double polyA sites and an inverse puromycin resistance cassette at the site of the start codon. The sgRNA vector and targeting vector were transfected into HeLa cells. After 24 h, cells were cultured in medium supplemented with 2 µg/ml puromycin for 2 weeks. Selected colonies were picked, and successful knockout was confirmed by RT-qPCR.

Gene ontology (GO) term analysis

Target mRNAs were analyzed by the PANTHER Overrepresentation Test (release 15-07-2016) (<http://pantherdb.org/>) using GO Ontology database released July 29, 2016. The Bonferroni correction for multiple testing was employed for statistical analysis.

Actinomycin D chase assay

For RNA stability measurements, HeLa cells were treated with 1 µg/ml actinomycin D (dissolved in DMSO [Sigma-Aldrich]). Samples were taken every 5–10 min, and RNA isolation, cDNA synthesis, and RT-qPCR were carried out as described previously (Tani *et al*, 2012).

BRIC followed by RT-qPCR

BRIC was performed as described previously (Tani *et al*, 2012; Imamachi *et al*, 2014). In brief, cells were incubated at 37°C in the presence of 150 µM bromouridine (BrU) (Wako Chemical, Tokyo, Japan) for 24 h in a humidified incubator with 5% CO₂. Cells were harvested at the indicated time points after replacing BrU-containing medium with BrU-free medium. Total RNA was isolated using RNAiso Plus (Takara), followed by isolation of BrU-labeled RNA using anti-BrdU mouse antibody (clone 2B1, MBL, Nagoya, Japan). The isolated RNA was used for RT-qPCR.

Glycerol gradient centrifugation

Whole-cell extracts were prepared by washing cells in PBS and scraping them into RSB100 buffer (100 mM NaCl, 10 mM Tris-HCl pH 7.4, 2.5 mM MgCl₂) containing 0.5% Triton X-100, 1% protease

inhibitor cocktail (Sigma-Aldrich), 5 µg/ml RNase A (Sigma-Aldrich), and 200 units/ml RNase T1 (Sigma-Aldrich). Cells were lysed and sonicated in a Bioruptor UCD-250 (Cosmo Bio, Tokyo, Japan), followed by centrifugation of the lysate at $20,000 \times g$ for 10 min at 4°C. Cleared supernatant (200 µl) was loaded onto a 2 ml 5–30% (v/v) glycerol gradient prepared in RSB100 buffer containing 0.5% Triton X-100 and 1% protease inhibitor cocktail. The gradient was centrifuged at $288,000 \times g$ in a SW41 rotor (Hitachi, Tokyo, Japan) for 3 h at 4°C, and 24 fractions were collected using a Gradient Fractionator (BioComp Instruments, New Brunswick, Canada).

Northern blotting analysis

Northern blotting was performed essentially as described previously (Sudo et al, 2016). The probes were labeled with biotin using Bio-Probe 3'-Oligonucleotide Labeling Kit with Bio-16-ddUTP (Enzo Life Sciences, Farmingdale, NY). Nucleotide sequences of ITS1 and ITS2 probes to detect pre-rRNAs were described previously (Hiraishi et al, 2018).

Expanded View for this article is available online.

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Author contributions

KI carried out most of the experiments. YF, TTay, and RO-M carried out microarray analysis. MK generated KO cells and performed infection studies using KO cells. GA and KT supported infection experiments. TY conducted imaging analysis. NI, AS, RN, and YS carried out NGS analysis and bioinformatics analysis. TTan and SM carried out Northern blotting. KI, AT, YI, MN, TY, THJ, and NA designed the study and wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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