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NusA-dependent transcription termination prevents misregulation of global gene expression

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Abstract

Intrinsic transcription terminators consist of an RNA hairpin followed by a U-rich tract, and these signals can trigger termination without the involvement of additional factors. Although NusA is known to stimulate intrinsic termination *in vitro*, the *in vivo* targets and global impact of NusA are not known because it is essential for viability. Using genome-wide 3′ end-mapping on an engineered *Bacillus subtilis* NusA depletion strain, we show that weak suboptimal terminators are the principle NusA substrates. Moreover, a subclass of weak non-canonical terminators was identified that completely depend on NusA for effective termination. NusA-dependent terminators tend to have weak hairpins and/or distal U-tract interruptions, supporting a model in which NusA is directly involved in the termination mechanism. Depletion of NusA altered global gene expression directly and indirectly via readthrough of suboptimal terminators. Readthrough of NusA-dependent terminators caused misregulation of genes involved in essential cellular functions, especially DNA replication and metabolism. We further show that *nusA* is autoregulated by a transcription attenuation mechanism that does not rely on antiterminator structures. Instead, NusA-stimulated termination in its 5′ UTR dictates the extent of transcription into the operon, thereby ensuring tight control of cellular NusA levels.

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Competing interests

The authors declare no competing financial interests.

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Author contributions

S.M. performed all experiments except for illumina library generation and sequencing. A.V.Y. discovered NusA-dependent termination *in vitro*. A.S. and I.A. processed raw sequencing data, mapped the sequencing reads to the genome and developed computational algorithms. P.B. and S.M. analysed the processed data. S.M. and P.B. wrote the manuscript. P.B. supervised the project.

Supplementary information is available online.

Regulation of bacterial transcription termination is not well understood. Whereas Rho-dependent termination depends on Rho, intrinsic terminators consist of an RNA hairpin followed by a U-tract and do not require additional factors for termination to occur[1]. The U-tract induces transcriptional pausing, which provides time for hairpin formation, leading to transcript release[2–4]. Uninterrupted RNA hairpins and U-tracts define canonical terminators, but some terminators contain imperfect hairpins or U-tracts, resulting in lower termination efficiency[5,6]. Although intrinsic termination does not require accessory factors, the conserved transcription elongation factor NusA can increase the termination efficiency *in vitro*[7–10]. Although it is assumed that NusA would also stimulate termination *in vivo*, because NusA is essential for viability[11,12], the targets, magnitude and consequence of stimulation are unknown. Using *Bacillus subtilis* as a model system, we used an engineered NusA depletion strain and a genomic 3′ end-mapping method to determine the effect of NusA on termination *in vivo*. We identified several terminators that completely depend on NusA for termination. Failure to terminate at these NusA-dependent terminators leads to misregulation of global gene expression. Our studies also revealed a transcription attenuation mechanism in which NusA autoregulates its expression via NusA-stimulated termination.

Construction of a NusA depletion strain

We engineered a *B. subtilis* NusA depletion strain (PLBS802) in which expression of NusA is repressed by withholding isopropyl β-D-1-thiogalactopyranoside (IPTG) from the growth medium (Supplementary Fig. 1). Growth of PLBS802 was slower in the absence of IPTG, an effect that was most prominent in rich Luria broth (LB) media (Supplementary Fig. 1). Primer extension indicated that the majority of *nusA* mRNA was rapidly depleted within 15 min of IPTG removal; ~1% of the mRNA was maintained as a consequence of leaky expression (Fig. 1a), while western blotting confirmed successful depletion of NusA (Fig. 1b). Because the level of NusA in the depletion strain when grown with 30 μM IPTG was comparable to the level in wild-type (WT) *B. subtilis*, this concentration of IPTG was used for all *in vivo* studies. Although a trace amount of NusA was present in cells grown without IPTG (Fig. 1b), to simplify the discussion we refer to the +IPTG condition as +NusA and the –IPTG condition as –NusA.

Identification of terminators by 3′ end-mapping

An RNA ligation-based high-throughput sequencing strategy was used to simultaneously identify termination sites at single nucleotide resolution and calculate termination efficiency. A unique RNA oligo was ligated to the 3′ end of rRNA-depleted total RNA isolated from PLBS802 grown ±IPTG (six biological replicates each) to mark the native 3′ ends, followed by illumina library preparation and sequencing (Supplementary Fig. 2). Whereas total reads were used for mRNA profiling, oligo-ligated sites precisely identified the 3′ ends of terminated, processed or partially degraded transcripts (Supplementary Table 1). Screening of these ends identified 2,130 possible intrinsic terminators; however, we considered 1,473 terminators with significant expression levels for the studies described below (Supplementary Table 2). Termination efficiency was determined at these terminators by comparing the number of terminated and readthrough reads at each point of termination

(Fig. 1c and Supplementary Fig. 2). NusA increased the termination efficiency of most terminators to some extent, with an average increase of 13.5% (Fig. 1d); however, the stimulatory effect of NusA ($\Delta\%T = \%T_{+\mathrm{NusA}} - \%T_{-\mathrm{NusA}}$) was generally much higher on weak terminators (Fig. 1e). Although most weak terminators were highly stimulated by NusA, the extent of stimulation at weak terminators was poorly correlated with basal terminator strength ($\%T_{-\mathrm{NusA}}$) (Fig. 1f, $r = -0.15$), indicating that NusA-mediated stimulation differs greatly among these terminators. NusA also reduced the termination of several terminators, although the extent of inhibition was very low in most cases (Fig. 1d).

Identification of NusA-dependent termination *in vivo*

Although we found a pronounced effect of NusA on weak terminators, the terminators differed greatly in the magnitude to which they were stimulated ($\Delta\%T$) (Fig. 1d,f). We grouped the terminators into four classes based on their response to NusA (Fig. 2a,b): NusA-independent (low $\Delta\%T$, for example, T_{rny} and T_{secY}); NusA-stimulated (moderate $\Delta\%T$, for example, T_{papB}); NusA-dependent (high $\Delta\%T$, for example, T_{metS}); and NusA-inhibited (negative $\Delta\%T$, for example, $TAtt_{thrS}$). NusA-independent terminators were subdivided into two groups based on basal termination efficiency ($\%T_{-\mathrm{NusA}}$) to distinguish between two mechanistically distinct terminator classes (Fig. 2a,b). Whereas strong NusA-independent terminators are always associated with small $\Delta\%T$ values because they have high basal termination efficiencies, NusA is ineffective at weak NusA-independent terminators despite low basal termination efficiencies. Strong NusA-independent terminators are the most abundant terminators in the *B. subtilis* genome, and weak NusA-independent and NusA-inhibited terminators are least abundant (Fig. 2c).

NusA-dependent terminators are particularly intriguing as they would greatly affect downstream gene expression depending on NusA availability (Table 1). Indeed, mRNA profiling indicated that the downstream genes are highly upregulated upon depletion of NusA (Supplementary Table 3). Because these terminators depend on NusA for efficient termination *in vivo*, they are not truly intrinsic in nature. Hence, these pseudo-intrinsic terminators constitute a new class of factor-dependent terminators and may represent the most critical NusA substrates. To determine whether a similar level of stimulation is observed *in vitro*, we tested several NusA-dependent terminators by *in vitro* transcription and found that stimulation was considerably lower than *in vivo* (Fig. 2d), indicating that *in vitro* transcription does not accurately measure the impact of NusA on termination *in vivo*.

Terminator features that affect their response to NusA

We aimed to test whether $\Delta\%T$ is correlated with terminator sequence by comparing the different terminator classes. The U-tracts of strong and weak NusA-independent terminators had the highest and lowest numbers of U residues, respectively (Supplementary Fig. 3). Whereas U was conserved at positions 1–5 in all classes, this feature was most prominent for strong NusA-independent terminators (Fig. 3a,b). NusA-stimulated terminators exhibited a high occurrence of U at the first two positions, while the prevalence of U at the second position was lower for NusA-dependent terminators. Both of these classes differed considerably from strong NusA-independent terminators in the conservation of U at

positions 3–6, with NusA-dependent terminators having the lowest conservation among these three classes (Fig. 3b and Supplementary Fig. 3). Weak NusA-independent terminators had the lowest conservation of U, while NusA-inhibited terminators exhibited intermediate conservation (Fig. 3b). The average Δ%*T* for all terminators containing a single U-tract interruption was highest when the non-U residue was at position 4 to 6 (Fig. 3c). Together, these findings indicate that NusA-dependency is correlated with a relatively high conservation of U at the first position and a gradual reduction in conservation at positions 2–6, suggesting that occupation of these positions by other bases favours stimulation by NusA.

We also investigated the relationship between Δ%*T* and the thermodynamic stability (Δ*G*) of terminator hairpins and found that NusA-dependent terminators have the weakest hairpins (Fig. 3d), suggesting that NusA compensates for weak hairpins by assisting with hairpin folding. To avoid bias associated with different U-tract variations, we examined the effect of Δ*G* on 357 terminators in which positions 1–7 were all U residues. A strong correlation was observed between weak terminator hairpins ($\Delta G > -10$ kcal mol^{-1}) and Δ%*T* (Fig. 3e,f), indicating that NusA stimulates terminators with weak hairpins.

As many terminators contain an A-tract upstream of the hairpin, we investigated whether they affect Δ%*T*. The finding that A-tracts are associated with strong NusA-independent terminators indicates that the A-tract contributes to basal terminator strength but not to Δ%*T* (Supplementary Fig. 3). An extended U-tract at positions 10 and 11 was also correlated with basal terminator strength, whereas the GC content of the stem (positions b1–b5) and the downstream element (positions 10–24) was not (Supplementary Fig. 3).

Weak terminators favour NusA-dependent termination

Our RNA-seq data indicated that NusA plays a vital role in the termination of weak non-canonical terminators, with the highest stimulation occurring at terminators containing a weak hairpin and/or distal U-tract interruptions. To test this model we performed a mutational analysis on the *B. subtilis trp* leader terminator (T_{trpL})[10]. Whereas NusA resulted in a modest increase in termination at WT T_{trpL} *in vitro*, NusA greatly stimulated the termination of each mutant terminator (Fig. 2e). Lowering Δ*G* with a hairpin mismatch, or the interruption of three distal U-tract residues, led to the highest degree of stimulation by NusA (Δ%*T*), consistent with our *in vivo* RNA-seq data. Moreover, combining the mismatch with the U-tract mutations resulted in severe termination defects, with termination being entirely dependent on NusA. We also found that *Escherichia coli* NusA was capable of exerting a similar effect at weak non-canonical terminators *in vitro*. As previously observed[9], NusA caused modest stimulation of the model tR2 intrinsic terminator, whereas the mutant terminators were highly stimulated by NusA, irrespective of the position of the mutations (Fig. 2f). When taken together with our *in vivo* termination studies, these results suggest that weak non-canonical terminators are critical NusA substrates in both Gram+ and Gram– organisms.

Examination of NusA-inhibited terminators *in vitro*

Although NusA can inhibit termination via antitermination complex formation at rRNA operons[13], none of the NusA-inhibited terminators that we identified were located in rRNA leaders, suggesting alternative mechanisms of inhibition (Supplementary Table 2). We therefore performed termination assays on four strongly inhibited terminators and found that all four terminators were actually stimulated by NusA *in vitro* (Fig. 2g). These results indicate that the terminators themselves are not responsible for NusA-mediated inhibition, suggesting that inhibition at these terminators *in vivo* depends on the sequence context surrounding the terminator and/or other unidentified factors.

NusA depletion leads to global gene expression defects

Depletion of NusA resulted in a striking change of gene expression throughout the genome. A total of 926 genes (22%) were significantly upregulated, and 730 (16%) were downregulated upon NusA depletion ($P < 0.05$), with expression of 466 (11%) and 238 (5%) being upregulated or downregulated more than twofold, respectively. Expression of genes downstream of NusA-dependent terminators was directly affected by increased readthrough upon NusA depletion (Fig. 4a), while NusA-stimulated and NusA-independent terminators also had substantial effects on downstream gene expression when preceded by highly transcribed genes (Fig. 4b). Furthermore, the absence of NusA resulted in increased antisense transcription at the junction of a number of convergent transcription units (Fig. 4c); failure to terminate in the absence of NusA caused increased antisense transcription at ~33% of the 580 convergent junctions in the *B. subtilis* genome, with ~14% exhibiting changes of several fold (Supplementary Table 1).

Interestingly, several operons exhibited apparent changes at the level of transcription initiation, as they were not preceded by a NusA-responsive terminator. For example, expression of the *frlBON* operon was completely repressed by NusA, whereas expression of the *comGA-GE* operon was completely dependent on NusA (Fig. 4d). Because NusA binds to elongating RNA polymerase (RNAP) after initiation, this is probably an indirect outcome of the direct effect of NusA on activators or repressors. We identified at least one such example where the arginine activator/repressor protein AhrC was upregulated fourfold upon NusA depletion by readthrough of a NusA-dependent terminator. In a probable indirect effect, the *rocABC* and *rocD-rocE-argI* operons, both of which are activated by AhrC[14], were upregulated via increased promoter activity (Supplementary Fig. 4). Further studies are required to explain the wide alteration of promoter activities throughout the genome upon NusA depletion.

Misregulation of replication and DNA metabolism genes

The effect of NusA depletion was most prominent on readthrough of NusA-dependent terminators (Fig. 4e), suggesting that regulation of downstream gene expression represents a general function of NusA-dependent terminators. Hence, we sought to determine whether NusA-dependent terminators regulate particular cellular processes by modulating readthrough. For this purpose, we considered genes that were upregulated at least twofold (*P*

< 0.05) as a direct consequence of termination failure (Supplementary Table 3). Gene ontology (GO) term analysis of these genes revealed high enrichment of terms related to DNA replication and DNA metabolism, and several other processes were affected to a lesser extent (Fig. 4f). Highly affected replication and DNA metabolism genes include *polC* (DNAP III α subunit), *dnaB*, *dnaD*, *dnaI* and *priA* (primosome), *recG* (DNA helicase), *recN* and *radA* (DNA repair), *nth* (endonuclease III), *disA* (DNA integrity scanning protein) and *smc* (chromosome condensation and segregation). mRNA profiling showed that each of these genes was overexpressed several-fold in the absence of NusA (Fig. 4g), indicating that depletion of NusA causes misregulation of replication and DNA metabolic pathways. Among the few regulatory factors that were affected, most notable was the RNAP pause-stimulating factor NusG[15,16], which was upregulated 2.5-fold as a direct consequence of termination failure (Supplementary Fig. 4).

Autoregulation of *nusA* expression

nusA is the second gene of the *ylxS–nusA–ylxR–rplGA–infB–ylxP–rbfA* operon driven by the σ^A-dependent P_{ylxS} promoter (Fig. 5a). 3′ end-mapping identified two NusA-stimulated terminators (T1 and T2) in the 5′ UTR of this operon, suggesting that the operon is regulated by transcription attenuation (Fig. 5a,b). The absence of overlapping antiterminator structures to block formation of either T1 or T2 suggested that NusA might control expression of the operon by modulating termination. Indeed, both T1 and T2 function as NusA-stimulated terminators *in vitro* and *in vivo* (Fig. 5c,d).

We also examined the expression of chromosomally integrated P_{ylxS}-5′ UTR-*lacZ* transcriptional fusions in which the 5′ UTR contained T1 and/or T2, as well as a fusion in which both terminators were deleted. Depletion of NusA resulted in a sixfold increase in expression of the fusion containing both terminators (Fig. 5e, T1T2). Expression of fusions containing only T1 or T2 indicated that T1 was the primary target of NusA-regulated termination (Fig. 5e), consistent with the RNA-seq data (Fig. 5d). In addition, expression of this operon is affected by termination at upstream NusA-dependent (T_{proS}, Fig. 4a) and NusA-stimulated (T_{polC}) terminators (Supplementary Table 2). It is also apparent that NusA depletion indirectly caused an increase of transcription initiation at P_{ylxS} (Supplementary Fig. 5). Together, these mechanisms constitute a complex autoregulatory circuit in which a homeostatic level of NusA is maintained in the cell.

Discussion

Depletion of NusA allowed us to assess the genome-wide effect of NusA on termination. Although our results indicate that NusA causes a slight stimulation at the majority of intrinsic terminators, it also led to the striking discovery that a large number of weak non-canonical terminators depend on NusA for efficient termination *in vivo*, indicating that this class of terminator is not truly intrinsic.

E. coli NusA consists of an N-terminal RNAP-binding domain, three RNA-binding domains (S1, KH1 and KH2), and two C-terminal autoinhibitory domains (AR1 and AR2), whereas *B. subtilis* and archaeal NusA lack the AR domains[17–19]. It is thought that NusA stimulates

termination by assisting with hairpin folding and/or by slowing elongation of RNAP[9,20,21]. Whereas the upstream U-tract residues are essential for initiation of DNA: RNA hybrid melting, the downstream U residues are responsible for slowing elongation[3]. Our studies showed that terminators with distal U-tract disruptions tend to be highly stimulated by NusA *in vivo*, suggesting that NusA is critical for slowing transcription within interrupted U-tracts. Our finding that weak NusA-independent terminators have the lowest conservation of U at positions 1 and 2 suggests that NusA does not readily compensate for the lack of U residues at these positions. It has also been suggested that NusA promotes hairpin folding, either directly by stabilizing the hairpins through contacts to duplex RNA[20], or indirectly by eliminating inhibitory interactions between RNAP and the hairpin[9]. Our observation that terminators with weak hairpins ($\Delta G > -10$ kcal mol^{-1}) tend to be NusA-dependent suggests that NusA directly assists with hairpin folding. Sequence features other than the hairpin and U-tract exerted little to no effect on NusA's ability to enhance termination.

Our RNA-seq studies also led to the surprising finding that NusA inhibits termination at ~5% of the intrinsic terminators *in vivo*, although inhibition was not observed with isolated terminators *in vitro*. Although it is not clear how NusA would inhibit these terminators, NusA may favour the formation of antiterminator-like structures in nascent transcripts because NusA is known to alter co-transcriptional RNA folding pathways[22]. The fact that 17/78 NusA-inhibited terminators are located in 5′ UTRs is consistent with this possibility (Supplementary Table 2). Further studies are required to define the mechanism by which NusA inhibits termination at these terminators *in vivo*.

Our results also establish that NusA regulates global gene expression directlyand indirectly by controlling transcription readthrough, particularly at NusA-dependent terminators. NusA-dependent terminators directly regulate genes involved in replication and DNA metabolism, suggesting that the contribution of NusA to the maintenance of genome stability extends beyond its role in transcription-coupled DNA repair[23]. However, changes in gene expression cannot be attributed solely to NusA-dependent terminators, as NusA-stimulated and even NusA-independent terminators contribute to altered expression of downstream genes when preceded by highly expressed genes.

The substantial increase of antisense transcription observed at several converging transcriptional units following NusA depletion is reminiscent of transcription defects caused by inhibition of Rho and NusG, which have been reported to suppress pervasive antisense transcription in *B. subtilis* and *E. coli*[24,25]. Perhaps the concerted action of Rho, NusG and NusA is required to minimize the synthesis of antisense transcripts, and inhibition of any of these components leads to increased antisense transcription. Further genomic studies involving NusA depletion in Rho and/or NusG knockout strains will be helpful in determining the individual and combined contributions of these factors in reducing pervasive transcription.

We also identified a complex autoregulatory circuit involving NusA-stimulated termination and regulation of promoter activity to maintain tight control of *nusA* expression in *B. subtilis* (Fig. 5 and Supplementary Fig. 5). A previous bioinformatics study reported that the 5′ UTR of *nusA* operons of sequenced bacterial genomes might contain a ubiquitous

transcription attenuator[26], although attenuation had never been verified for any organism until now. Thus, the *B. subtilis* NusA autoregulatory circuit may be a highly conserved mechanism that operates in many bacterial species. This homeostatic control mechanism would minimize fluctuations of intracellular NusA levels in response to cellular or environmental changes. Quantification of RNAP and NusA in exponentially growing *B. subtilis* revealed a 1.6-fold excess of NusA, suggesting that there is sufficient NusA for most transcribing RNAPs to be bound[27]. What, then, is the significance of NusA in modulating termination efficiency? The importance of NusA lies in the fact that many imperfect terminators are not self-sufficient. In this respect, NusA increases the number of functional terminators in the genome and allows fine-tuning of gene expression. Therefore, NusA probably relaxes the selective pressure on intrinsic terminators to maintain a canonical-like structure.

Methods

Library preparation and data analysis

Six replicates of the NusA depletion strain (PLBS802) were grown in minimal-ACH medium (0.2% acid-hydrolysed casein and 0.5% glucose) with 12.5 μg ml^{-1} tetracycline ±30 μM IPTG. RNA was isolated, and barcoded illumina libraries were generated from oligo-ligated transcripts (see Supplementary Information). Equal amounts of the libraries were pooled and 100 × 100 paired end sequencing was performed with an Illumina HiSeq 2500 in rapid run mode. Raw data were processed using standard procedures. Mapping of 3′ ends and calculation of termination efficiencies were performed using custom Python algorithms (see Supplementary Information).

Primer extension

PLBS802 was grown at 37 °C in LB with 100 μM IPTG and 12.5 μg ml^{-1} tetracycline. Cells were harvested in exponential phase, washed and resuspended in LB lacking IPTG, and growth was resumed. Cells were harvested at various times and RNA was isolated as described[28]. Primer extension using a ^{32}P end-labelled primer (5′-GAGAGCATCTAATAATTCACTGC-3′) was performed as described[28].

Western blot

PLBS802 was grown at 37 °C in minimal-ACH medium with 12.5 μg ml^{-1} tetracycline and 0–100 μM IPTG and harvested in exponential phase. Protein samples (1 μg) were fractionated in a 12% SDS gel and transferred to a 0.2 μm nitrocellulose membrane. Purified his-tagged NusA, σ^A and cell lysates were probed with rabbit anti-NusA or anti-σ^A antibodies (1:5,000 dilution) and developed using enhanced chemiluminescence following incubation with HRP-conjugated goat anti-rabbit antibody (GenScript).

In vitro termination

Single-round *in vitro* transcription reactions and data analysis [10,16]. For other of WT and mutant T_{trpL} terminators were performed as described *B. subtilis* terminators, 12-nt halted transcription elongation complexes were formed by withholding CTP. Elongation was resumed by the addition of all four NTPs together with heparin ±1 μM NusA. Similar

reactions were performed with WT and mutant λtR2 terminators, except that *E. coli* RNAP and NusA were used. Electrophoresis and quantification of radiolabelled transcripts followed published procedures[10].

β-galactosidase assay

Derivatives of *B. subtilis* PLBS802 containing P_{ylxS}-5′ UTR-*lacZ* transcriptional fusions integrated into the *thrC* locus were grown at 37 °C in minimal-ACH medium with 12.5 μg ml^{-1} tetracycline ±30 μM IPTG. Cells were grown to exponential phase, and β-galactosidase activity was determined as described[29].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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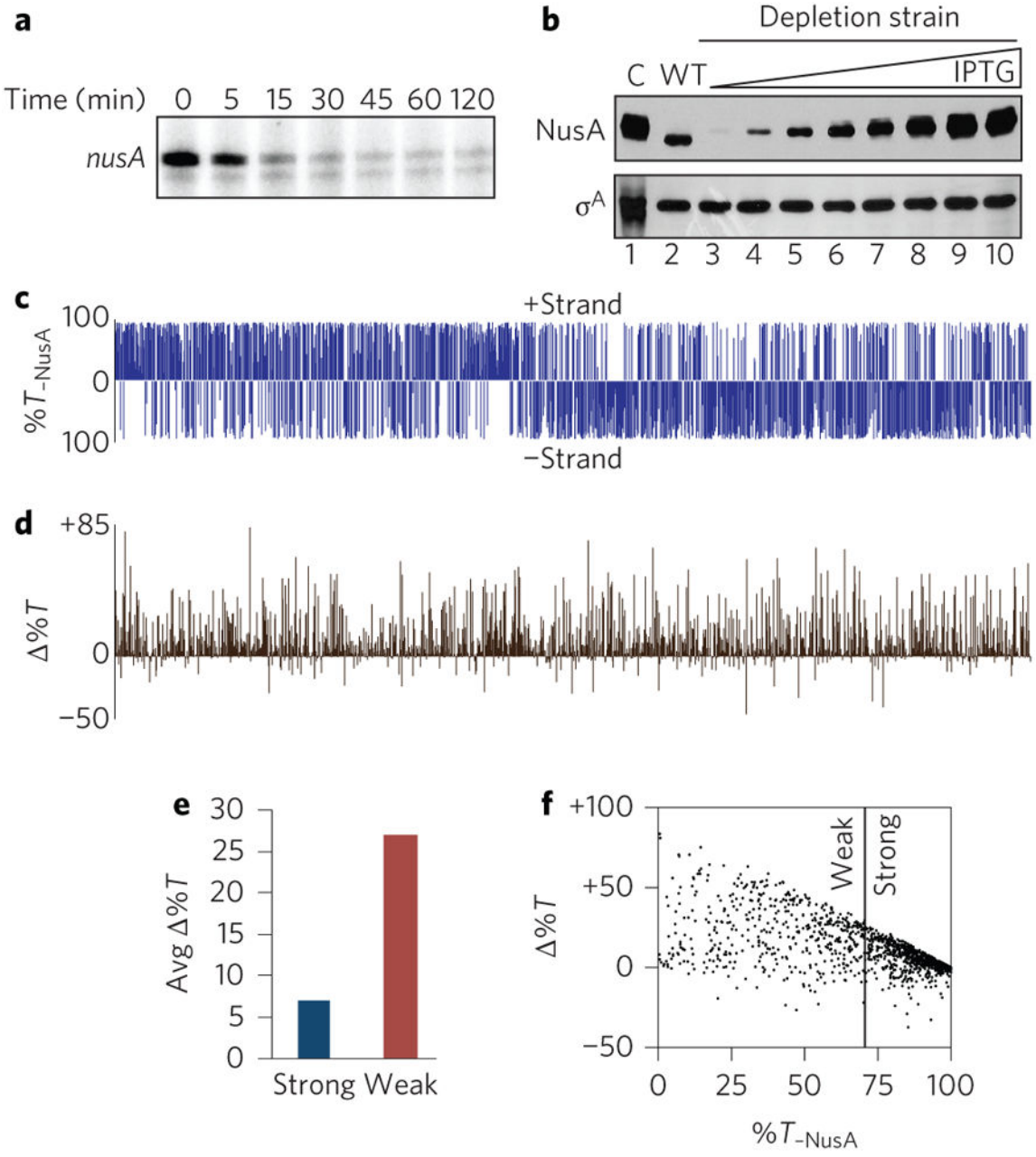

Figure 1. Depletion of NusA and its effect at intrinsic terminators *in vivo*

a, Primer extension assay to monitor *nusA* mRNA depletion. PLBS802 was grown with IPTG to exponential phase, washed, and growth was then resumed without IPTG for the indicated times before RNA extraction. This is a representative gel of an experiment that was performed twice. The entire gel is shown in Supplementary Fig. 6a. **b**, Western blot confirming NusA depletion. PLBS802 was grown to exponential phase without (lane 3) or with IPTG (10–100 μM, lanes 4–10; lane 6 is 30 μM). Lane 1: purified $NusA_{6His}$ and σ^A (sigma factor). Lane 2: protein lysate from WT *B. subtilis*. This is a representative blot of an experiment that was performed three times. The entire blot is shown in Supplementary Fig. 6b. **c**, Terminators identified throughout the genome (not to scale) showing basal termination

efficiency (%$T_{-\mathrm{NusA}}$). **d**, Effect of NusA on termination efficiency (Δ%T). **e**,**f**, Effect of NusA on strong and weak intrinsic terminators.

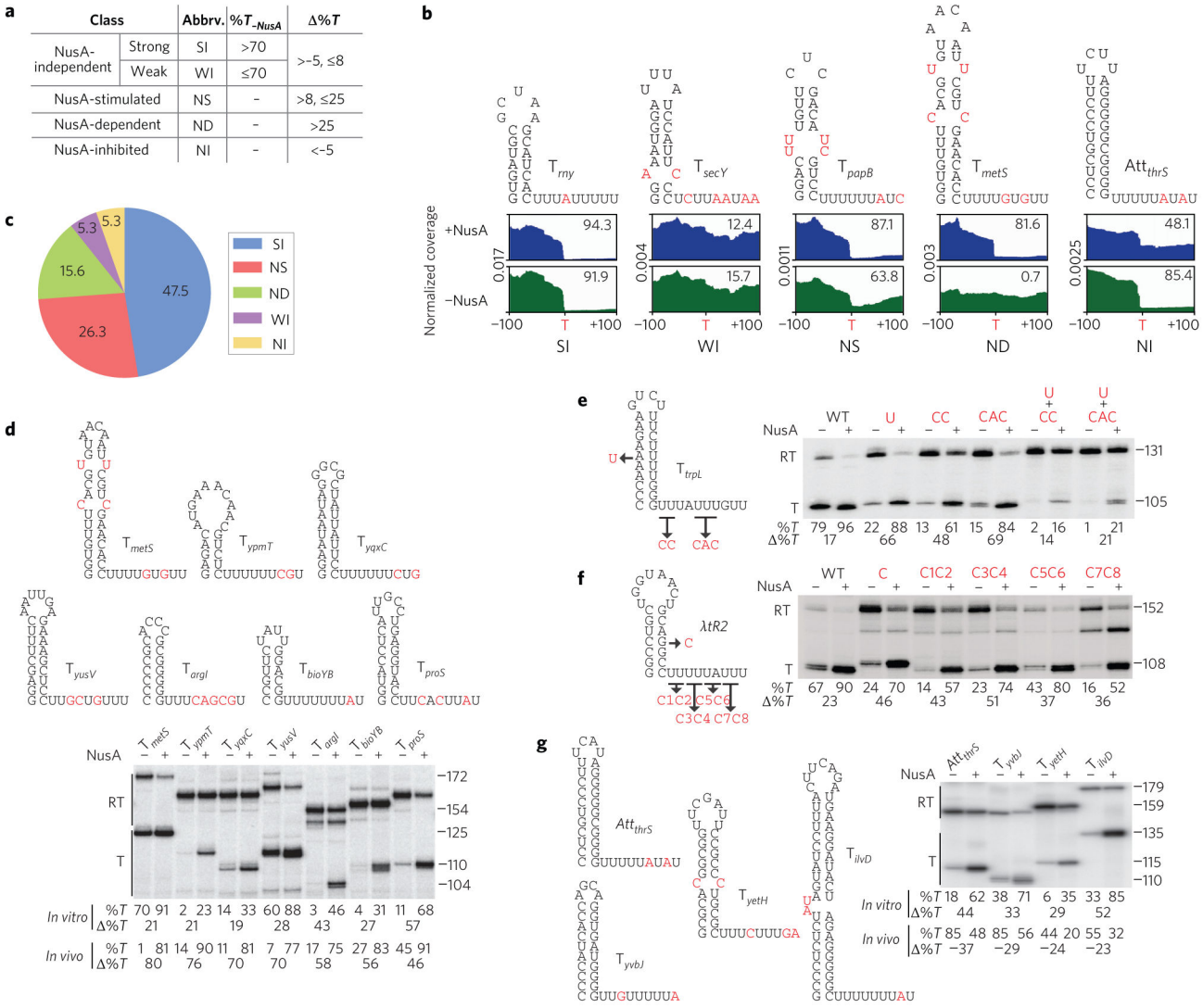

Figure 2. Effect of NusA on intrinsic termination *in vivo* and *in vitro*

a, Classification of the terminators based on their response to NusA *in vivo*. **b**, Examples of the five terminator classes. Bases deviating from a canonical terminator are in red. Normalized coverage ±NusA are in blue and green, respectively. Termination efficiency (%*T*) is shown in each box; *y*-axes show normalized coverage, described in detail in the Supplementary Information. **c**, Percentage of different terminator classes in the *B. subtilis* genome. **d**, *In vitro* effect of NusA on NusA-dependent terminators identified *in vivo*. Terminators were tested two (*ypmT*, *yqxC*), three (*metS*, *yusV*, *argI*, *bioYB*) or six (*proS*) times. This is a representative gel. The entire gel is shown in Supplementary Fig. 6c. **e**, WT and mutant *trpL* terminators. This is a representative gel of an experiment that was performed three times. The entire gel is shown in Supplementary Fig. 6d. **f**, WT and mutant tR2 terminators. This is a representative gel of an experiment that was performed three times. The entire gel is shown in Supplementary Fig. 6e. **g**, NusA-inhibited terminators identified *in vivo*. This is a representative gel of an experiment that was performed twice. The entire gel is shown in Supplementary Fig. 6f. In **d**–**g**, single-round *in vitro* transcription

termination assays were performed with *B. subtilis* RNAP and NusA (**d**,**e**,**g**) or *E. coli* RNAP and NusA (**f**). Termination efficiencies (%*T*) and the effect of NusA (Δ%*T*) are shown for each terminator. Positions of terminated (T) and readthrough (RT) transcripts are marked. Molecular sizes of transcripts are shown on the right of each gel.

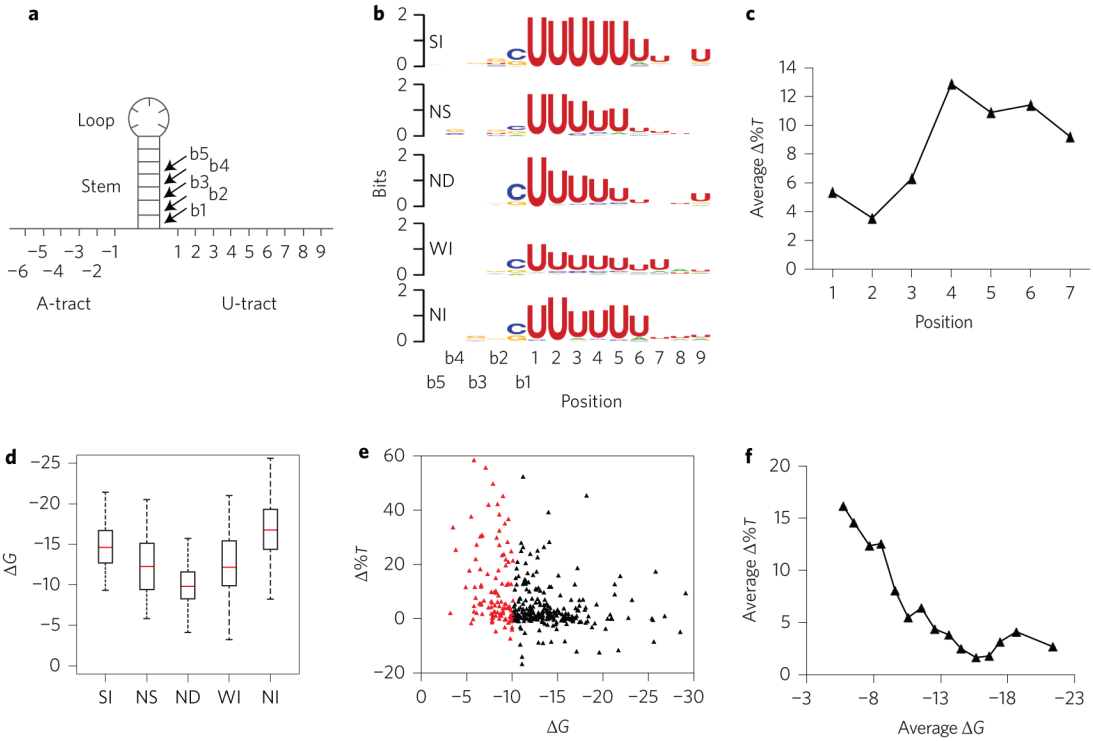

Figure 3. Terminator features contributing to stimulation by NusA

a, Diagram of an intrinsic terminator. Bases of the A-tract (–1 to –6), bottom of the stem (b1 to b5) and U-tract (1 to 9) are marked. **b**, Sequence logo showing the conservation of U residues in U-tracts of different terminator classes. **c**, Average Δ%*T* plotted as a function of the position of a single non-U base in the U-tract. **d**, Box plot of hairpin strength (Δ*G*, kcal mol^{-1}) in different terminator classes (Fig. 2a). The whiskers represent maximum and minimum Δ*G* values for each class, the red midline shows the median and boxes represent the first through the third quartiles. **e**, Scatterplot of 357 terminators with U at position 1–7 of the U-tract. Δ%*T* plotted as a function of Δ*G*. **f**, Average Δ%*T* plotted as a function of average Δ*G* for terminators described in **e**. Data in **b** and **d** were generated from the 50 strongest SI terminators in the absence of NusA, the 50 WI least affected by NusA, the 50 NS and 50 ND most stimulated by NusA and the 35 NI most inhibited by NusA.

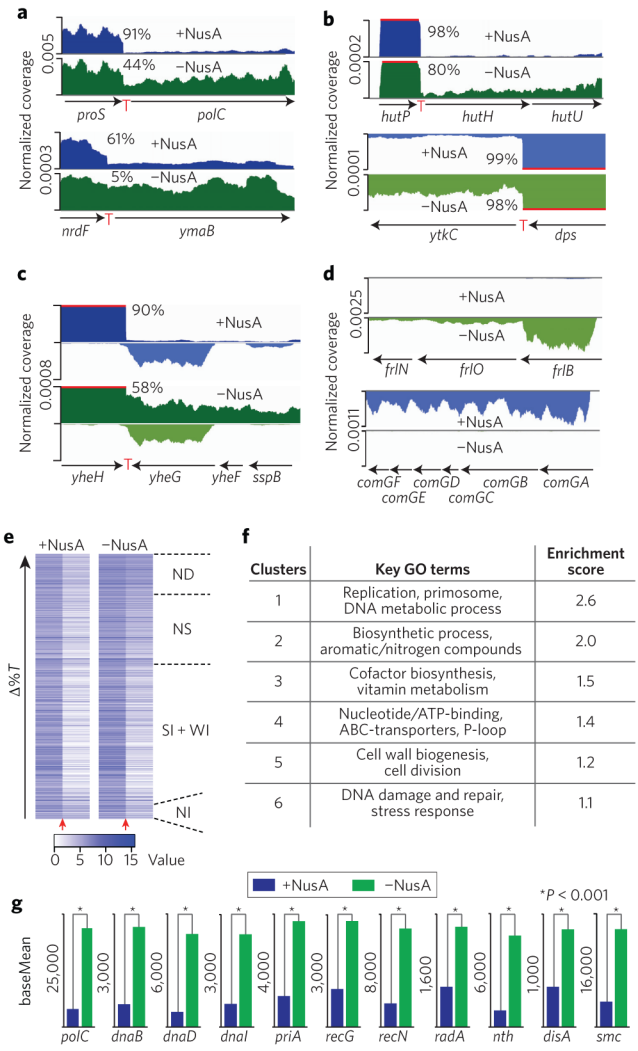

Figure 4. NusA depletion alters expression of genes downstream of intrinsic terminators

a, Overexpression of *polC* and *ymaB* as a direct consequence of readthrough of NusA-dependent terminators. **b**, Overexpression as a direct consequence of readthrough of NusA-stimulated (*hutHU*) and strong NusA-independent (*ytkC*) terminators when preceded by highly expressed genes. **c**, Increased expression of antisense RNA by readthrough of a NusA-dependent terminator. **d**, Indirect NusA-mediated repression (*frlBON*) or activation (*com*). Intrinsic terminators are not present upstream of the *frl* and *com* operons. In **b**,**c**, red bars indicate that the coverage is off scale. **e**, Heat map of readthrough transcription. Lines represent transcription units arranged by descending Δ%*T*. Average coverage of 10-nt windows upstream and downstream of the point of termination (red arrowhead). **f**, GO-term enrichment analysis of genes directly regulated by NusA-dependent terminators. Enrichment scores above 1.3 are highly significant. **g**, Increased expression of replication and DNA metabolism genes regulated by NusA-dependent termination.

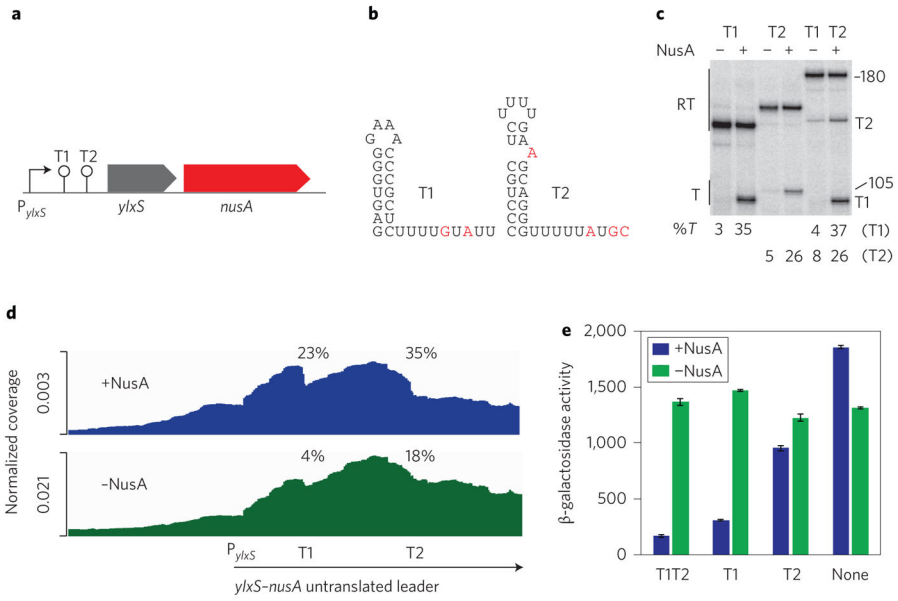

Figure 5. Autoregulation of *nusA* expression by NusA-mediated transcription attenuation

a, *ylxS–nusA* region showing positions of T1 and T2. **b**, NusA-stimulated terminators T1 and T2, with residues deviating from canonical intrinsic terminators in red. **c**, Single-round *in vitro* transcription termination assays showing the effect of NusA on termination at T1 and/or T2. Termination efficiencies (%*T*) are shown for T1 and T2. Positions of terminated (T) and readthrough (RT) transcripts are marked. Molecular sizes of transcripts are shown on the right of the gel. This is a representative gel of an experiment that was performed three times. The entire gel is shown in Supplementary Fig. 6c. **d**, Effect of NusA on termination at T1 and/or T2 *in vivo*. Scales for normalized read counts differ by sevenfold, indicating that expression is much higher without NusA. **e**, β-galactosidase activity (Miller units) of *ylxS–lacZ* transcriptional fusions containing T1 and/or T2. Strains were grown ±30 μM IPTG to exponential phase. Error bars reflect the standard error of four independent experiments.

Table 1

Top ten NusA-dependent terminators.

Terminator	$\%T_{+NusA}$	s.e.m.	$\%T_{-NusA}$	s.e.m.	$\Delta\%T$	*P*-value*
T_{ydjM}	84	2.6	1	4.4	83	1.6×10^{-8}
T_{metS}	82	1.6	1	3.7	81	3.2×10^{-9}
T_{ypmT}	90	3.9	15	2.7	75	2.5×10^{-8}
T_{yqxC}	81	1.5	11	3.1	70	2.2×10^{-9}
T_{yuxK}	77	2.7	7	9.7	70	2.1×10^{-5}
T_{yusV}	77	2.2	7	1.3	70	1.3×10^{-10}
T_{yfzA}	82	3.0	18	7.7	64	2.6×10^{-5}
T_{ytzE}	86	1.8	22	4.7	64	1.6×10^{-7}
T_{nrdR}	94	1.8	31	5.9	63	2.6×10^{-6}
T_{xre}	76	4.2	15	2.4	61	1.5×10^{-7}

**P*-values were calculated from six replicates using a two-tailed *t*-test.