CHAPTER V
Suppressor Mutations in Rho for N function
5.1 Introduction
We have been working towards understanding the mechanism of N-mediated antitermination at Rho dependent terminators in all the previous chapters. The same can be greatly appreciated if a Rho mutant, capable of overcoming N and terminating even in its presence can be obtained. Although Rho is an essential gene in *E. coli* (Brown *et al.*, 1981), viable Rho mutants have been isolated in which Rho activity is modified. One such class of rho mutants was reported to prevent the growth of a certain λ phage, λr32 (Simon *et al.*, 1979, Das *et al.*, 1983; Washburn *et al.*, 1996) that carries an IS2 insertion of a Rho-dependent terminator, between tR1 and the cII gene, and is consequently more dependent on the λN antitermination protein than λ (Das *et al.*, 1983). The mutant Rho referred as rho026 and later identified as P103L Rho (Washburn *et al.*, 1996), caused interference with the growth of λr32 through inhibition of N-mediated antitermination (Das *et al.*, 1983; Patterson *et al.*, 1994).

We isolated a mutation in Rho which could suppress N function. The mutant was E134K. A different mutation at this position (E134D) had earlier been reported (Friedman *et al.*, 1994). We also constructed the Rho mutant P103L, reported earlier and studied it along with the E134K Rho protein. The *in vitro* studies of E134K Rho mutant helped in comprehending how in the absence of N, the terminator Rho engages NusA to form a termination competent configuration (Peters *et al.*, 2011), while when N is present, these factors are utilized for the antitermination process (Santangelo *et al.*, 2011).

5.2 Material and methods
5.2.1 Random mutagenesis and screening of suppressors in Rho
pRS649 containing the WT rho gene was transformed into XL1-Red mutator strain (Stratagene; Muteeb *et al.*, 2010) to obtain the mutagenised rho plasmid library in the similar way as described for N gene. The mutagenised Rho library was electroporated into the strain RS1017 (P<sub>lac-</sub> nutR/tR1-trp<sup>+</sup>–lacZYA) containing pRS668 with WT H-19B N gene and the transformants were directly plated on MacConkey agar plates supplemented with 1% (w/v) lactose and appropriate antibiotics to get dominant Rho mutants in the presence of the WT copy in the chromosome. Alternatively, the chromosomal copy of rho gene of the transformants was removed by P1 transduction, and then plated. Rho mutants that can suppress N function appeared as pink/white colonies on these plates. The putative Rho mutant plasmids were isolated and re-transformed into the background strain for
ensuring the mutant phenotypes. The plasmids were subsequently sequenced to confirm the mutations.

5.2.2 Cloning and Protein purification of Rho mutants- P103L and E134K

P103L Rho was obtained by Site Directed Mutagenesis, using RS415 as forward primer and RS416 as reverse primer. The SDM was done on pRS96 (Appendix I, His tagged Rho cloned in pET 21b vector) by following the methodology described in QuickChange™ XL site-directed kit (Stratagene). PCR products were subjected to DpnI digestion to digest the parental plasmid and transformed into DH5α ultra competent cells. Plasmids isolated from the transformants were sequenced to confirm the mutations. E134K Rho was obtained during Random Genetic Screening. It was subcloned from pCL1920 vector into the Ndel/XhoI sites of pET21b plasmid to obtain Non His tagged E134K for protein purification. Sub cloning was done using DeepVent proofreading enzyme. The desired mutation was confirmed by sequencing.

pET21b plasmid with His tagged P103L Rho cloned into it was transformed into the BL21(DE3) over expressing strain. Protein expression in BL21 (DE3) was done by inducing at OD_{600} of 0.5 with 1 mM IPTG for 3 hrs. at 32 °C. His tagged Rho was purified using Ni-NTA column. The cells were collected by centrifuging the cell culture at 10000 rpm for 5 min. and were then resuspended in lysis buffer (pH 8.0) containing 100 mM NaH_{2}PO_{4}, 10 mM Tris-HCl (pH 8.0), 300 mM NaCl, 10 mM imidazole, 1 mg/ml Lysozyme and 50 μg/ml PMSF. After ressuspending the cells thoroughly, Lysozyme was added and the contents were incubated on ice with constant stirring for 30
min. The cells were sonicated till solution turned yellowish and complete lysis took place. The cells were centrifuged after lysis at 4 °C; 12000 rpm for 30 min. Supernatant was transferred to prechilled fresh tube. The Ni-NTA beads were packed in column that works under gravity and the column was equilibrated with 5 bed volumes of lysis buffer. The supernatant was applied to the column (typically 5 ml bed volume) and the unbound fraction was allowed to flow through and collected for analysis. The column was washed with 10 bed volumes of wash buffer (pH 8.0) containing 100 mM NaH$_2$PO$_4$, 10 mM Tris-HCl (pH 8.0), 300 mM NaCl and 20 mM imidazole. The protein was eluted with 30 ml elution buffer composed of 100 mM NaH$_2$PO$_4$, 10 mM Tris-HCl (pH 8.0), 300 mM NaCl and 500 mM imidazole. Various fractions were then checked on 10% SDS-PAGE (Figure 5.1). After dialyzing with TGED [10 mM Tris-HCl (pH 7.8), 0.1 mM EDTA, 0.1 mM DTT and 5% (v/v) glycerol] with 0.1 M NaCl, the protein was further loaded on heparin-sepharose column (Amersham). The bound protein was eluted with increasing ionic strength (Figure 5.1b). Pure protein was dialysed for 10 hr. against storage buffer [20 mM Tris-HCl (pH 7.9), 0.1 mM EDTA, 0.1 mM DTT, 100 mM KCl and 5% (v/v) glycerol] with three buffer changes and concentrated using Amicon YM100 (Millipore). Glycerol to the final concentration of 50% (v/v) was added to the concentrated pure protein and it was distributed into aliquots of 100 μl and stored at -70 °C.

E134K Non His tagged Rho was purified by passing the cell lysate over a heparin–agarose column and purified by batch elution method with high salt, which yielded a greater than 95% pure protein (Figure 5.2). E134K Rho protein was expressed in BL21 (DE3) over expression strain. Cells were induced at 0.5 OD$_{600}$ with 1 mM IPTG for 3 hr. at 32 °C and the cell pellet was collected as described above. The pellet was resuspended in grinding buffer [0.05 M Tris-HCl (pH 7.8), 2 mM EDTA, 0.1 mM DTT, 1 mM 2-mercaptoethanol, 0.2 M NaCl, 5% (v/v) glycerol and 50 μg/ml PMSF]. Lysozyme was added to the final concentration of 1 mg/ml. The mix was incubated on ice for about 20 min. 0.05% sodium deoxycholate was then added to the mix and incubated on ice for an additional 30 min. The sample was sonicated for 6 min thrice or till complete lysis occurred. The lysate was collected after spinning the sonicated sample at 12000 rpm for 30 min. and was dialysed against TGED buffer [10 mM Tris-HCl (pH 7.8), 0.1 mM EDTA, 0.1 mM DTT, 5% (v/v) glycerol] with 100 mM NaCl for about 12 hr. with three buffer changes. The protein was loaded on Heparin column and the bound protein was
eluted via a step gradient with increasing NaCl concentrations. The fractions were checked for the presence of protein by running on a 12% SDS-PAGE. The fractions containing pure Rho protein were pooled and concentrated in storage buffer [10 mM Tris-HCl (pH 8.0), 0.1 mM DTT, 0.1 mM EDTA, 100 mM KCl] with 5% (v/v) glycerol by using Amicon YM 100 (Millipore). After concentrating the sample to about 3 ml, the protein was dialysed against storage buffer with 50% (v/v) glycerol.

**Figure 5.2: Purification of Non His tag E134K Rho mutant protein**

(a) Induction of E134K Rho protein. M, Marker; UI, Uninduced sample; I, Induced sample P, Pellet; L, lysate (b) Purification through Heparin column by batch elution with NaCl gradient. M, Marker; Lo, Load; F, Flow through. E134K Rho eluted at 0.55 - 0.65 M NaCl. The 50 and 100 kDa marker bands are marked with ʻ*ʼ.

### 5.2.3 In vitro characterization of Rho mutants

#### 5.2.3.1 Characterization of Rho mutants through continuous transcription termination assays

Linear DNA templates for continuous in vitro transcription assays was made by PCR amplification from pRS22 (pT7A1-H-19B nutR-tR1) using oligos RS58/RS RK23B (Figure 5.3, 5.4, 5.8). Reactions were performed in the transcription buffer (T buffer; 25 mM Tris-HCl, pH 8.0, 5 mM MgCl₂, and 50 mM KCl) at 32 °C. The reactions were initiated with 10 nM of DNA template, 40 nM RNA polymerase, 175 μM ApU, 5 μM each of GTP and ATP and 2.5 μM of CTP to make a 23-mer EC. [α-³²P] CTP (3000 Ci/mmole) was added to the reaction to label the EC₂₃. The complex was chased with either 20 μM or 250 μM NTPs in presence of 10 μg/ml rifampicin for 15 min at 32°C. 50 nM WT or mutant Rho, 200 nM NusG, 300 nM NusA and 100 nM WT or mutant H-19B N were added to the chase solution. The reaction was stopped by extracting with phenol after 15 min. of incubation at 32 °C followed by precipitation in ethanol in the presence of glycogen. RNA pellets were then re-suspended in form amide loading dye and loaded
onto an 8% or 6% sequencing gel, exposed to a phosphor imager screen (Amersham) and scanned by Typhoon 9200 Phosphorimager.

5.2.3.2 Characterization of Rho mutants through transcription termination assays on stalled EC

For studying RNA release from stalled Elongation Complex, transcription reaction with T7A1- nutR/tR1-lacO template was done under the same conditions as described above, but in this case, DNA was immobilized on the streptavidin-coated magnetic beads and lac repressor was added to about 100 nM, before chasing the 23-mer EC (Figure 5.5, 5.11). For RNA release assays, reactions were chased for two minutes, washed once, followed by the addition of Rho in the presence of 1 mM ATP. The reaction was incubated at 32°C for different time points and half of the supernatant was taken out for the "S" lanes and the rest was phenol extracted and used for the "S+P" lanes (Figure 5.5, 5.11). Fraction of RNA released was estimated as, \( \frac{[S]}{([S] + [P])} \) and plotted against time using SIGMAPLOT software. The data points for all the curves, were fitted to the equation of an exponential curve, \( y = a*[1-exp (-\lambda*x)] \), where \( \lambda \) gives the rate and "a" gives the amplitude. The \( r^2 \) values for each of the fittings were 0.99 or above (Dutta et al., 2008; Kalarickal et al., 2010).

5.2.3.3 Characterization of Rho mutants through ATPase assay

ATPase activity of the WT and mutant Rho protein was measured from the release of inorganic phosphate (Pi) from ATP after separating on the polyethyleneimine TLC sheets (Merck) with 0.75M KH2PO4 (pH 3.5) as the mobile phase. In all the ATPase assays, the composition of the reaction mixture was 25 mM Tris–HCl (pH 8.0), 50 mM KCl and 5 mM MgCl2, 1 mM DTT and 0.1 mg/ml of BSA. For ATPase assay with poly(C), 50 nM Rho was incubated with 1 mM ATP, together with \([\gamma-32P] \) ATP (3500 Ci/mmol; BRIT, India) at 37 °C and ATP hydrolysis was initiated by the addition of 20 \( \mu \)M poly (C). Aliquots were removed and mixed with 1.5 M formic acid at various time points. Release of Pi was analysed by exposing the TLC sheets to a Phosphor-imager screen for 5 min. and subsequently by scanning using Typhoon 9200 (Amersham) and the intensities of ATP and Pi were quantified by Image QuantTL software (Figure 5.10B). The initial rates of the reaction were determined by plotting the nano moles hydrolysed ATP versus time using linear regression method. The slope of the plot gives ATPase activity of Rho in terms of nmols of ATP hydrolysed per minute. This value of slope was divided by
amount of Rho present in μg, to obtain final rate of ATP hydrolysis as nmol of ATP/min/μg Rho (Dutta et al., 2008, Chalissery et al., 2007).

5.2.3.4 Characterization of Rho mutants through Gel shift assays
RNA binding to the primary RNA-binding site of the WT and mutant Rho proteins was measured by gel retardation using an endlabeled rC25 oligo (Figure 5.10A). 10 nM of labelled oligo was used with the WT or mutant Rho for the binding assays in the transcription buffer [25 mM Tris-HCl (pH 8.0), 5 mM MgCl2, 50 mM KCl, 1 mM DTT and 0.1 mg/ml of BSA] supplemented with 10% glycerol and 1 mM AMPPNP. We maintained the hexameric form of Rho in the nanomolar concentration range by adding the ATP analogue, 5’-adenylyl-β,γ-imido-diphosphate (AMPPNP). The reactions were performed at 37 °C for 10 min. before loading onto gradient native PAGE (8% to 4%) casted in 0.5× TBE (Tris–boric acid–EDTA) buffer. Electrophoresis was also performed in 0.5× TBE buffer in cold. Gels were then dried and analyzed by Phosphorimager. RNA binding to radio-labelled H-19B cro RNA (Figure 5.10A), was also measured for WT and E134K rho, in the same way as described above.

5.2.3.5 In vitro pull down of the Rho–NusG and Rho-NusA complex
For pull-down of Rho-NusG complex (Fig. 5.19B), 30 μg of the His tagged NusG (full length or NusG-CTD) and 6 μg of the non-His-tagged Rho (WT or E134K) were mixed in 100 μl of lysis buffer (pH 8.0) containing 100 mM NaH2PO4, 100 mM NaCl, 10 mM imidazole and PMSF; and incubated at 37 °C for 10 min. Protein mixture was added up to 100 μl of pre-equilibrated Ni-NTA beads and incubated at room temperature for 10 min. The supernatant was removed after spinning at 2000 rpm for 2 min. The beads were then washed with 100 μl of wash buffer (100 mM NaH2PO4, 100 mM NaCl, and 50 mM imidazole), and the proteins were eluted with 100 μl of elution buffer composed of 100 mM NaH2PO4, 100 mM NaCl, and 500 mM imidazole. Samples were loaded on 15% SDS-PAGE and stained by coomassie blue. For pull –down of Rho-NusA complex (Fig 5.9A), 30 μg of the His tagged NusA and 6 μg of the non-His-tagged Rho (WT or E134K) were taken, keeping other conditions same and samples were loaded on 12% SDS-PAGE followed by coomassie blue staining.
5.3 Results

5.3.1 Isolation of suppressor mutations in Rho

We looked for suppressor mutations in Rho which enabled it to overcome N. A unique mutation in Rho, E134K, which was able to terminate in presence of WT N, was isolated. We also constructed another Rho mutant, P103L, which was reported to prevent the growth of a certain λ phage, λr32 (Simon et al. 1979, Das et al., 1983; Washburn et al., 1996).

5.3.2 Suppression of N by Rho mutants

5.3.2.1 Suppression of N by Rho mutants in continuous transcription assays

We checked the in vitro transcription termination behaviour of the two Rho mutants with respect to WT Rho, as well as the antitermination ability of H-19 B N in their presence by performing continuous transcription assays on the linear DNA template pT7A1-H-19B N nutR/tR1.

5.3.2.1.1 The template sequence used for continuous transcription assays

The template used for continuous transcription assay was amplified from plasmid pRS22, the sequence of the region of interest of which is shown (Figure 5.3).

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Primer RS58 ----→ T7A1 Promoter
1  CCAGGAATTG GGGAT
61  ACTTAAAGTC TAACCTATAG GATACCTACA GCCATCGAGA GGGACACGGG CGAAGCTTG
121 CGAATCGAAT CGAATAAGCT ACTCGTAATT TCACGGTTTA TAAACCATGAAAAGAA
181 ACCTAGGTGT CGTATGCCGT AGAGCAGGCT CGTGCATAAGT AACACGCTA TTTCAGCAAT
241 GGACATTCGT CCTAGC TGACAAACGAG AGTCCCGAATAA TATCTGACCA ACTAAGGCA
291 BoxA
301 TATGCGTTGC CACGCATTTG TTTCAACTAG CTATTCAACTA TNAAAATCT TAAGAAATGG
361 AACAAACAAAG TTACACAGAA CTATCACAGC GAGAAATGGG CTGGCGCGGA ACTGATTAC
421 TCATCAACCT GTCAACGCTT ACCACCGGAG AAGACGAGCA AA GATGATTGGCTGTCATGA
481 TCACTCGGTT TATGGGTTTA TAGCTTCGGT CTTGTGTGCT TTTGGCAGGC
541 ATCAGACTAG CAGAAGCTGC TGGAGTGGTA TAGGTGTCTG TTTGGCAGGC
541 ATCAGACACC CGGTTGCGCG ATCTCTCTAGA GTCGAGTCTGGCAAAC CAACTAC

Figure 5.3: Sequence of a portion of plasmid pRS22

The single Rho dependent terminator template pT7A1-nutR/tR1 was amplified from plasmid pRS22 using RS58 and RSRK23B primers. The position of primers, promoter T7A1, transcription start site (ATC) and H-19B boxA and boxB region is marked. The U-less tract is highlighted in green.
Forward primer RS58, upstream of promoter T7A1, and reverse primer RSRK-23B were used for template amplification. On this template, the full length Run Off product, from the transcription start site (ATC) to the position of downstream primer RSRK23B is 500 nucleotide in length while termination by WT Rho results in a much shorter transcript.

5.3.2.1.2 Deductions from continuous transcription assay

The extent to which the Rho mutants could suppress the N mediated antitermination was measured on the H-19B nutR/tR1 terminator template. Transcription was carried out in the presence/ absence of N and the two Nus factors, NusA and NusG, along with either WT or a mutant Rho protein. The Rho mutants, E134K and P103L showed early termination phenotype compared to the WT Rho (Figure 5.4). E134K induced termination even earlier, almost close to the WT Rho binding site ("spacer region").

Figure 5.4: In vitro suppression of N by Rho mutants

Autoradiogram showing the in vitro transcription assays at H-19B nutR/tR1 terminator under indicated conditions. Termination zones in presence of WT and mutant Rho are indicated by two headed arrows and also with dotted lines. The locations of nutR boxA, spacer and boxB on the RNA are indicated next to the gel. Amounts of run-off transcript in each lane are indicated below the gel. The concentrations of Rho (WT/ mutant) and H-19B N were 50 nM and 25 nM, respectively. The assay was done at 25 μM NTPs. The template used to perform the continuous transcription assay is indicated above the gel.
WT N could antiterminate efficiently to give the full length RO product in presence of WT Rho (5.4ure), but the amount of run-off (RO) product, was reduced by 2-fold when P103L was present, while it was reduced by more than 5-fold in case of E134K (compare the %RT values shown at the bottom of figure 5.4). Early termination phenotype of P103L was consistent with an earlier report (Washburn et al., 1996). Therefore, the Rho mutants; E134K and P103L, are the suppressors of the N function.

5.3.2.2 Suppression of N by Rho mutants on a stalled Elongation Complex
As E134K Rho could terminate in the spacer and boxB region of nut/tR1, we hypothesised that the unusual early termination property of E134K observed in continuous transcription assays could arise from its binding to a region in nascent RNA further upstream to the nutR/rut site. In such a case, E134K Rho might be suppressing N by initiating termination before N could loads onto the nut boxB RNA. In order to test this, we measured the RNA release efficiency of E134K from a stalled EC already modified with N (Figure 5.5). If indeed E134K is suppressing N function by not allowing it to access its binding site nut boxB, then in the case where N is bound to EC beforehand, any effect of E134K mutation in Rho should not be observed, that is, it should not suppress N function.

We stalled the EC artificially in the nut/tR1 termination region using lac repressor as a roadblock and then investigated the RNA release efficiency of E134K Rho in presence/absence of N. EC23 was first made and then chased in presence/absence of N to make the RB.

5.3.2.2.1 Template used to form a stalled EC
The template used to perform RNA release assays from stalled EC was prepared from plasmid pRS22 using forward biotinylated primer RS83 and reverse primer RS404, with lac operater site. This template strand of DNA was biotinylated at its 3’ end while the lac operator sequence was present at its 5’ end.

5.3.2.2.2 Deductions from the study of RNA release kinetics at Road Blocked EC
The release of RNA from stalled EC in the presence of Rho + 1mM ATP was observed at different time points within a 5 min. window. In the absence of Rho, no RNA release was observed up to 5 minutes, the maximum duration of observation (Figure 5.5, 0’ lane
stands for ‘zero Rho’). The rate of RNA release by E134K was much slower compared to that in presence of WT Rho (Figure 5.5). Interestingly, presence of WT N did not have any significant effect on this E134K mediated RNA release rate as it was not able to prevent RNA release by E134K even from the stalled EC (Figure 5.5; compare with the profiles in the presence of WT Rho shown). Therefore, E134K inhibited N function, even when N could interact with its binding site on RNA- the nut boxB. As EC-bound N-NusA complex did not prevent the action of E134K Rho, initiating an early termination by this Rho may not be the mechanism for overcoming N.

![Figure 5.5: Suppression of N by E134K Rho on a stalled EC](image)

Autoradiogram showing the amount of RNA released by E134K Rho, both in the absence or presence of WT H-19B N from the stalled EC formed on the T7A1-nutR/tR1-lacO template (above). Concentrations of E134K Rho and H-19B N were 50 nM and 100 nM, respectively. ‘S’ denotes half of the supernatant, and ‘P’ denotes the rest of the sample. RNA release was estimated as, \([2S]/([S]+[P])\) and plotted against time (below) both in the absence or presence of WT H-19B N. RNA release by WT Rho on the same template is indicated by solid and dashed curves only. 300 nM NusA and 200 nM nusG were present.

### 5.3.2.3 Detection of binding site of E134K rho on nascent RNA by foot printing

The continuous and kinetic transcription studies of E134K Rho in presence and absence of N protein clearly revealed that it could suppress the N function of antitermination, even in a scenario, where N was first of the two proteins, among Rho and N, to be added in the experiment and to interact with nut RNA. Therefore, although E134K has an early termination phenotype, the information derived from functional assays revealed that initiating an early termination by this Rho may not be the mechanism for overcoming N. Detecting the binding site of E134K rho on nascent RNA by foot printing was the next step in understanding how E134K suppresses N antitermination.
5.3.2.3.1 RNAse T1 mediated footprinting for detection of binding site of E134K
We employed RNase T1 footprinting to probe the binding site of the E134K Rho mutant both in the presence of and absence of N on the nascent RNA (Figure 5.7). RNase T1 is an endoribonuclease that cleaves single-stranded RNA after guanine residues, on their 3' end. The presence of the Guanine residues in boxA, spacer and boxB ensured that useful information could be obtained from T1 cleavage (Figure 5.6).

![Figure 5.6: Sequence of the nutR region of H-19B](image)

The boxA and boxB sequences are included inside a box. The sequence separating boxA and boxB is called the ‘spacer’. A part of spacer, encircled in green, forms a stem-loop structure, as shown. The entire boxB sequence forms a hairpin loop.

5.3.2.3.2 Deductions from footprinting studies
The stalled EC at the lac operator site of the template described (Figure 5.7) was first formed, and it was incubated with either WT or E134K Rho proteins complexed with the ATP analogue AMPPNP. The hexameric form of Rho in the nano-molar concentration range is maintained by adding ATP analogue AMPPNP. The non-hydrolyzable analogue of ATP was used as Rho may dissociate the EC in the presence of ATP. We had already performed extensive foot printing studies with WT Rho on this nascent RNA (Figure 4.15, 4.16).

The foot-printing profile revealed that like WT Rho, E134K also binds to the spacer region (Figure 5.7, lanes 4 and 6) but the pattern was significantly different. E134K induced T1 sensitivity to the two base paired G residues present in the stem part of the spacer hairpin (Figure 5.6, Figure indicated at the left side of the figure 5.7) which meant that this stem was melted upon binding to E134K Rho mutant.
Figure 5.7: RNase T1 foot-printing in the presence of E134K Rho

Foot-printing of the end-labelled nascent RNA of the stalled EC (RB) by RNase T1 in the presence of WT or E134K Rho. Protections on RNA by Rho or Rho+ NusA/G are indicated by dashed lines. Different regions of the nutR/r141 are also indicated by the side of the gel.

This sensitivity to T1 also meant that E134K did not protect this hairpin and its footprint was shifted downstream (indicated at the right side of the autoradiogram). Footprinting in presence of N-NusA/G complex showed that E134K Rho co-occupies the nut/rut site with N and NusA, but they exhibited a weaker protection at the spacer hairpin (Figure 5.7, lanes 8 and 10). We did not observe any footprint of E134K outside the spacer region. This indicated that E134K does not overcome N merely by binding upstream to the nut site. Since NusA is known binds to spacer (Prasch et al., 2009), and it was also shown during footprinting studies in the previous chapter (Figure 4.16), we propose that E134K induced melting of the spacer hairpin might have affected NusA binding as well as the N-NusA interaction at the nut site which in turn impaired N function. It has been proposed earlier that this spacer hairpin stabilizes the N-NusA interaction (Neely et al., 2000).

5.3.3 Unusual dependence of E134K Rho on NusA

The slow rate of RNA release by E134K Rho from a stalled EC (figure 5.5) suggests that it may have termination defect and its early termination behaviour in the presence of NusA/NusG (figure 5.4) indicates its dependence on these factors. Moreover, the E134K
mediated melting of spacer hairpin might interfere with NusA binding to nut site (Figure 5.7). As NusA and NusG are the integral part of N-antitermination, we hypothesized that this apparent Nus factor dependence of E134K might have impaired N-NusA interaction and hence we studied its properties in more detail.

5.3.3.1 Influence of NusA and NusG on in vitro transcription termination of Rho mutants

Knowing the transcription termination defects of Rho mutants, we proceeded to understand their in vitro transcription termination behavior. We used two separate templates, nutR/tR1 and trpt', fused to the T7A1 promoter (Figures 5.8A and B) and investigated the in vitro termination efficiency of E134K in the presence and absence of NusA and NusG to understand the nature of the Nus factor dependence.

Figure 5.8: Early transcription termination by Rho mutants

Autoradiograms showing the steady state single round in vitro transcription termination in the presence of WT/E134K Rho on H-19B nutR/tR1 terminator (A) and on trpt' terminator template (B). Termination region is indicated by dotted lines next to the transcript bands. RO denotes the run-off product. The concentration of Rho, NusA and NusG were 50 nM, 300 nM and 200 nM, respectively. The assay was carried out at 25 μM NTPs.

In case of WT Rho, presence of NusA delays the termination window whereas NusG induces early termination and an intermediate effect is observed in the presence of both of them (lanes 2-5 of 5.9A and 15-18 of 5.9B; Burns et al., 1998). In the absence of any factor, E134K Rho showed termination defects on both the terminators (increase in the
amount of RO; lane 6 of 5.9A and lane 2 of 5.9B). Unlike WT Rho, NusG on its own was unable to induce early termination or improve the efficiency of E134K Rho (lane 8 of 5.9A and lane 13 of 5.9B). Interestingly instead of delaying the termination, NusA improved the termination efficiency and together with NusG made E134K extremely efficient and exhibited early termination phenotype (lane 7, 9 of 5.9A; lanes 12, 14 of 5.9B). As E134K does not bind upstream of the \textit{rut} site (Figure 5.7), the early termination induced by NusA arose from the increase in the speed of RNA release.

\subsection*{5.3.3.2 In vitro NusA and NusG binding property of Rho mutant}

Since NusG on its own was unable to influence the \textit{in vitro} termination of E134K, while NusA had a very different effect on it, we hypothesized that it might be possible that E134K Rho does not bind to NusG and has acquired an unusual property of NusA-binding. We tested the \textit{in vitro} binding of NusA and NusG to E134K Rho by pull-down assays (Figure 5.9). A mixture of WT or E134K Rho with WT NusA protein was passed through Ni-NTA agarose beads and different fractions were loaded onto 12\% SDS-PAGE and visualized by Coomassie blue staining. Amounts of Rho and NusA used were 6 μg and 30 μg, respectively. Neither any unusual E134K-NusA interaction (as WT Rho does not bind to NusA) nor any defect in E134K-NusG complex formation (as WT Rho binds to full length NusG as well as to NusG-CTD) was observed (Figure 5.9A and B). However, NusA may influence different rate-limiting steps of the termination process (Shashni \textit{et al.} 2012) by interacting with E134K at the \textit{rut} site.

![Figure 5.9: In vitro pull-down assays of Rho](image)

A) \textit{In vitro} pull-down assays of Rho with His-tagged WT NusA. FT: flow-through fraction from the column; W: washed fraction; E: eluted fraction from the column B) \textit{In vitro} pull-down assays of His-tagged WT NusG (FL or CTD) with WT or E134K Rho. Different fractions with the same notations as in (A) were loaded onto 15\% SDS-PAGE.
5.3.3.3 Primary and Secondary RNA-binding properties of the E134K Rho

Continuing with our efforts to understand the functional properties of E134K Rho, we tested the primary and secondary RNA binding as well as RNA dependent ATPase activities of E134K.

![Figure 5.10: Primary and Secondary RNA-binding properties of the E134K Rho](image)

**Figure 5.10: Primary and Secondary RNA-binding properties of the E134K Rho**

 Autoradiograms of a gradient native PAGE showing the migrations of the free and Rho-bound rC25 oligo and H-19B cro RNA. rC25 is a 25 mer poly(C) RNA and H-19B cro RNA contains the tR1 terminator sequence from the lambdoid phage H-19B. Free and bound fractions of the RNA are indicated. Binding events were performed in the presence of ATP analogue, AMPPNP to maintain the hexameric status of Rho. In all the experiments, RNAs were labelled with P32. The concentration of Rho is indicated. Concentration of labelled oligo was 10 nM. B) ATPase assay of Rho in the presence of poly(C) as RNA cofactor. Representative plots showing the amount of release of inorganic phosphate (Pi) from [γ-32P] ATP with time. The data were fitted by linear regressions using SIGMAPLOT. The average values (from two measurements) of the rates of Pi release in the presence of WT or E134K Rho are indicated. Rates of ATP hydrolysis are indicated as nmol/min/μg of Rho. C) Location of E134 on the dimeric structure of Rho shown as red sphere. Primary RNA binding domain is in yellow and the P, Q and R-loops are in blue ribbons.

Primary RNA-binding property of the mutant Rho protein was measured by gel-shift assays, using a short RNA, rC25, and a natural RNA from H-19B phage having the nut/rut site (Kalarickal et al. 2010). E134K showed comparable affinity with respect to
WT Rho on the shorter RNA but a significantly weaker RNA binding on the longer RNA which passes through the secondary RNA binding sites (H-19B RNA; Figure 5.10A). Measurement of ATPase activity demonstrated that E134K Rho has a significantly slower ATP hydrolysis rate with the strong Rho-substrate polyC (Figure 5.10B). The ATPase activity of E134K was found to be more than two fold lesser than that of WT Rho. This suggested that the E134K Rho mutant is defective in secondary RNA binding. This defect is also consistent with the location of the E134K mutation on the Rho structure, which is near the path of the RNA in the central hole (Figure 5.10C).

### 5.3.3.4 Effect of NusA on RNA-release efficiency of E134K Rho mutant from stalled elongation complex

E134K showed a secondary RNA binding defect, a slow rate of RNA release from stalled EC, a poor transcription termination phenotype in continuous transcription assays, and a NusA dependent improvement in this termination during continuous transcription. Therefore, we hypothesised that the defect in secondary RNA binding may give rise to a slow rate of RNA release by E134K from a stalled EC and NusA might be able to improve the RNA release rate, just like it aids E134K to terminate better during *in vitro* continuous transcription assays.

We stalled the EC artificially in the *nut/rI1* termination region using lac repressor as a roadblock and then investigated the RNA release efficiency of E134K Rho in presence/absence of NusA. EC23 was first made and then chased in presence/absence of NusA to make the RB. The release of RNA from stalled EC in the presence of Rho + 1mM ATP was observed at different time points and the effect of NusA on RNA release kinetics of E134K and WT Rho was determined. While NusA improved the rate of RNA release of E134K by about 2-fold (Figure 5.11B), its effect on WT Rho was quite the opposite (Figure 5.11C). Instead of any increase, the RNA release efficiency of WT Rho was found to decrease slightly in the presence of NusA. Hence, NusA improves the E134K termination efficiency by increasing its rate of RNA release and this might have stimulated NusG function indirectly (Figure 5.8A and B, lanes 9 and 18). Presence of NusA very close to Rho at the *nut/rut* site might have helped E134K to properly bind the RNA in its secondary binding site(s) as well as to speed-up its isomerization steps leading to the translocase competent state.
Figure 5.11: Effect of NusA on RNA-release efficiency of E134K Rho from a stalled elongation complex

A) Lac repressor was used to stall the EC in the nutR/tR1 termination region, 198 nt away from the boxB hairpin. Fractions of RNA released by E134K B) and WT Rho C) are plotted against time both in the absence or presence of NusA from the stalled EC inside H-19B nutR/tR1 termination region.

5.4 Discussion

Involvement of NusA in Rho-dependent termination has been implicated in different reports (Zheng et al., 1994; Burns et al., 1998; Cardinale et al., 2008, Saxena et al., 2011), but the role of NusA in this process is still unknown. Here for the first time, we report a Rho mutant, E134K, whose function is highly dependent on NusA. Interestingly, while the mutant itself remained many fold defective for termination, it could effectively suppress N antitermination.

Continuous transcription assays revealed that the E134K mutation in Rho could suppress the N mediated antitermination at Rho dependent terminator nutR/tR1 and decrease the amount of full length Run Off product by 5- fold, compared to that which was formed in presence of WT Rho and N (Figure 5.4). An early transcription termination phenotype of
E134K (Figure 5.4), with it terminating almost close to the WT Rho binding site, "spacer" region was also confirmed. As E134K Rho depicted an early termination property, there was a possibility that its binding to a region in nascent RNA further upstream to the nutR/rut site was suppressing N by initiating termination before the latter could load onto the nut boxB RNA. However, the RNA release efficiency of E134K from a stalled EC already modified with N (Figure 5.5), revealed that presence of WT N did not have any significant effect on the E134K Rho mediated RNA release rate from the stalled EC (Figure 5.5). This was in contrast to the effect of N observed on the RNA release kinetics of WT Rho. Therefore E134K was able to suppress N function, even in a case where N could interact with its binding site on RNA- the nut boxB. The information derived from the functional assays revealed that initiating an early termination by this Rho may not be its mechanism for overcoming N. Further attempts to understand the mode by which E134K Rho was suppressing N antitermination gave many interesting insights. Similar to WT Rho, E134K was found to bind to the nascent RNA at the spacer region, but the pattern of its footprint was significantly different than that of WT Rho as the stem part of the spacer hairpin was found to be melted upon binding to E134K Rho mutant (Figure 5.7). Moreover, no footprint of E134K outside the spacer region was detected. This again indicated that E134K does not overcome N merely by binding upstream to the nut site. The in vitro transcription termination behaviour of E134K in the absence of any factor showed that it was termination defective, and unlike WT Rho, NusG on its own was unable to induce early termination while NusA improved the termination efficiency instead of delaying it (Figure 5.8). Interestingly, NusA together with NusG made E134K extremely efficient and it exhibited early termination phenotype. However, neither any unusual E134K-NusA interaction nor any defect in E134K-NusG complex formation was observed in vitro (Figure 5.9). The difference between the WT and E134K Rho was however observed in the secondary RNA binding with the ATPase activity of E134K being more than two fold lesser than that of WT Rho (Figure 5.10). Another difference between the mutant and the WT was the way NusA affected the RNA release process caused by them from the stalled EC. While NusA improved the rate of RNA release of E134K by about 2-fold, its effect on WT Rho was quite the opposite (Figure 5.11).

The unusual dependence of the E134K Rho on NusA for efficient termination (Figure 5.8) and its suppression activity of N function (Figures 5.4, 5.5), led us to propose that
Rho and N competes for the same NusA molecule. N-NusA interaction removes NusA from the Rho-dependent termination pathway and makes the latter process less efficient. NusA improves the termination efficiency of E134K by increasing the rate of RNA release and stimulating the NusG function. We suggest that the secondary RNA binding defect of E134K (Figure 5.10) is rectified by NusA-mediated chaperoning of the RNA into the secondary channel and the stabilization of RNA in the central hole may also stimulate the NusG function. While isolation of E134K mutant of Rho brought an understanding of the importance of NusA in this regard, an attempt was made to understand the importance of NusG in this respect in the next chapter.