

# Npl3, a new link between RNA-binding proteins and the maintenance of genome integrity

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**Abbreviations:** RBP, RNA-binding protein; mRNP, messenger ribonucleoprotein particle; RF, replication fork; DDR, DNA damage response; hnRNP, heterogeneous nuclear ribonucleoprotein; NPC, nuclear pore complex; SR, Ser-Arg-rich; DSB, double-strand break; RRM, RNA-recognition motif; CTD, carboxy-terminal domain; RNAPII, RNA polymerase II; Ser2P, phosphorylation of Serine 2; CBC, cap-binding complex; 4NQO, 4-nitroquinoline 1-oxide; UV, ultraviolet light; MMS, methyl-methane sulfonate; HU, hydroxyurea; HR, homologous recombination; NHEJ, non-homologous end joining; NER, nucleotide excision repair; TC-NER, transcription-coupled nucleotide excision repair

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**T**he mRNA is co-transcriptionally bound by a number of RNA-binding proteins (RBPs) that contribute to its processing and formation of an export-competent messenger ribonucleoprotein particle (mRNP). In the last few years, increasing evidence suggests that RBPs play a key role in preventing transcription-associated genome instability. Part of this instability is mediated by the accumulation of co-transcriptional R loops, which may impair replication fork (RF) progression due to collisions between transcription and replication machineries. In addition, some RBPs have been implicated in DNA repair and/or the DNA damage response (DDR). Recently, the Npl3 protein, one of the most abundant heterogeneous nuclear ribonucleoproteins (hnRNPs) in yeast, has been shown to prevent transcription-associated genome instability and accumulation of RF obstacles, partially associated with R-loop formation. Interestingly, Npl3 seems to have additional functions in DNA repair, and *npl3Δ* mutants are highly sensitive to genotoxic agents, such as the antitumor drug trabectedin. Here we discuss the role of Npl3 in particular, and RBPs in general, in the connection of transcription with replication and genome instability, and its effect on the DDR.

## Introduction

In eukaryotic cells, transcription and translation occur in different cellular

compartments. Whereas DNA is transcribed to mRNA in the nucleus, protein synthesis takes place in the cytoplasm, implying that the mRNA has to be exported for translation. This export occurs through a macromolecular complex embedded in the nuclear membrane termed the nuclear pore complex (NPC).<sup>1</sup> Transcription and export are coupled with the processing of pre-mRNAs into mRNAs, which includes 5'-end capping, splicing, and 3'-end cleavage and polyadenylation. During these processes, the mRNA is co-transcriptionally packaged by a number of RNA-binding proteins (RBPs) leading to the formation of an export-competent messenger ribonucleoprotein particle (mRNP).<sup>2</sup> The most abundant classes of RBPs in eukaryotes are the heterogeneous nuclear ribonucleoproteins (hnRNPs) and the mammalian serine-arginine-rich (SR) proteins. Both protein families carry out different functions from pre-mRNA packaging to splicing, mRNA export and translation.<sup>3,4</sup>

One of the most abundant hnRNPs in *Saccharomyces cerevisiae* is Npl3, which is also an SR-like protein and shares structural homologies with both protein families.<sup>5,6</sup> Npl3 is a multifunctional protein that participates in a variety of RNA-related processes, including transcription, splicing, mRNA export, and translation.<sup>7-10</sup> Additionally, a recent report has provided evidence of an Npl3 role in preventing genome instability.<sup>11</sup> The function of Npl3 at transcribed genes is necessary to avoid R-loop formation between the nascent mRNA and the transcribed

DNA strand, thus protecting the genome against harmful transcription–replication collisions. The sensitivity of *npl3Δ* cells to double-strand break (DSB)-inducing genotoxic agents, including the antitumor drug trabectedin, suggests a role of Npl3 in the DNA damage response (DDR) and provides new evidence on the importance of RBPs in the maintenance of genome integrity and cancer prevention that we discuss here from a global perspective.

### Npl3, a Multifunctional Protein Mediating RNA Export

Npl3 was initially identified as a protein required for the localization of nuclear proteins (nuclear protein localization 3) and for pre-rRNA processing.<sup>12,13</sup> However, it was soon described for its role as an mRNA export factor and its ability to shuttle between the nucleus and the cytoplasm together with the mRNAs.<sup>8,14</sup> Npl3 shares structural homologies with hnRNPs and SR proteins. It has 2 RNA-recognition motifs (RRMs), a conserved RNA-binding domain shared by a number of RBPs from yeast to humans (Fig. 1), and an N-terminal domain composed of RGG tripeptides and RS dipeptides that confer

to Npl3 a high RNA-binding capacity.<sup>5</sup> There are 2 other SR-like proteins in *Saccharomyces cerevisiae*, Gbp2 and Hrb1, which also function in mRNA export. In contrast to Npl3, Gbp2 and Hrb1 physically associate with the THO complex, a key nuclear RNA binding factor connecting transcription, mRNP biogenesis, and genome instability.<sup>15</sup> In addition, Npl3 promotes pre-mRNA splicing, while Gbp2 and Hrb1 are involved in quality control of spliced mRNAs.<sup>9,16</sup>

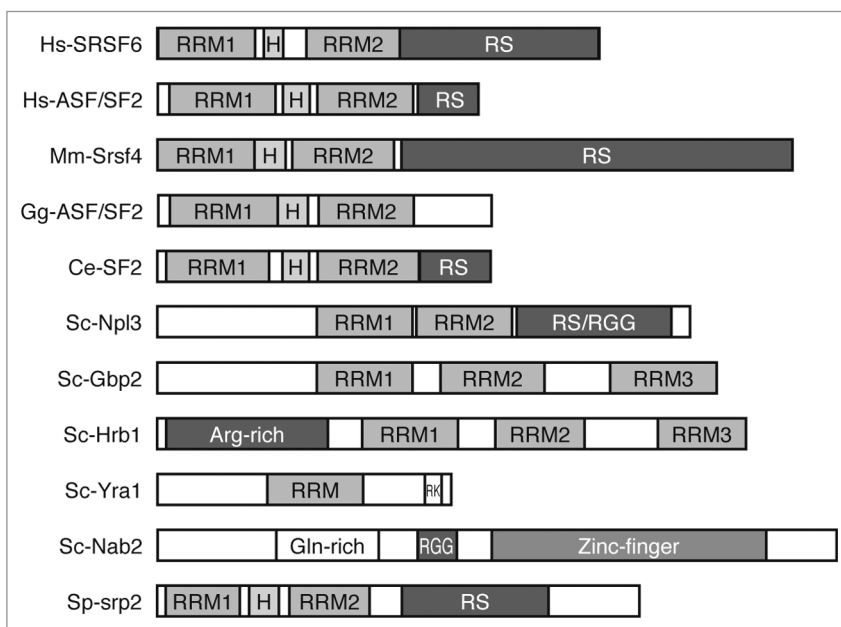
Npl3 binds to genes in a transcription-dependent manner by its physical interaction with the C-terminal domain (CTD) of RNA polymerase II (RNAPII) at the phosphorylated Ser2,<sup>7,17</sup> which is a mark of transcription elongation. Binding of Npl3 to RNAPII promotes transcription elongation and inhibits termination by competing with the CF1A cleavage and polyadenylation complex.<sup>18</sup> This mechanism is believed to avoid premature termination at cryptic termination sites and serves as a self-regulation mechanism of Npl3 due to the existence of a weak alternative poly-A site downstream of the *NPL3* gene.<sup>19</sup> The anti-termination activity of Npl3 is progressively reduced during transcription elongation by casein-kinase Cka1-dependent phosphorylation,

allowing the action of termination factors.<sup>7</sup> Then, mRNA 3'-end processing stimulates Npl3 dephosphorylation by Glc7 phosphatase, which causes Npl3 binding to the nascent mRNA and promotes its export to the cytoplasm.<sup>20</sup> Consistent with this model, Npl3 is distributed genome-wide throughout the length of transcribed ORFs in an increasing gradient toward the 3' end and decreasing again around the poly-A site,<sup>11</sup> not easily observed in another study.<sup>21</sup> The increasing accumulation of Npl3 toward the 3' end of genes correlates with that of Ser2P of the RNAPII CTD, with which Npl3 physically interacts.<sup>7,22,23</sup> Indeed, the THO complex and several transcription elongation factors also bind to active genes in a 5' to 3' gradient.<sup>23–25</sup> Therefore, it is possible that the RNAPII CTD participates in recruiting Npl3 during transcription elongation.

After export to the cytoplasm, Npl3 is phosphorylated by Sky1 and released from the mRNA.<sup>26</sup> Indeed, purification of the mRNPs by pull down with different RNA-binding tagged-proteins has revealed that Npl3 is a component of the nuclear mRNPs, but not of the cytoplasmic mRNPs, such as the Cap-binding complex (CBC) proteins Cbp20 and Cbp80, the THO subunit Thp2, the RNA export factor Yra1, the export receptor Mex67, the NPC-associated THSC/TREX-2 subunit Sac3, and the nucleoporin Mlp1.<sup>27,28</sup> These observations indicate that Npl3 is bound to the mRNA at early stages of mRNP biogenesis and remains associated until it reaches the cytoplasm, where it is dephosphorylated and reimported to the nucleus.

### A Role of Npl3 in the Maintenance of Genome Integrity

Improper mRNP biogenesis and export leads to defects in transcription and mRNA export and transcription-associated genome instability.<sup>29</sup> Part of this instability is mediated by the accumulation of R loops, consisting on a DNA–RNA hybrid and the displaced non-template DNA strand. R loops form naturally during specific cellular processes, such as *Escherichia coli* plasmid replication, mitochondrial



**Figure 1.** RNA-binding domains in different eukaryotic RBPs. RRM, RNA-recognition motif; H, hinge domain; RS, Arg-Ser-rich; RGG, Arg-Gly-Gly-rich; RK, Arg-Lys-rich; Hs, *Homo sapiens*; Mm, *Mus musculus*; Gg, *Gallus gallus*; Ce, *Caenorhabditis elegans*; Sc, *Saccharomyces cerevisiae*; Sp, *Schizosaccharomyces pombe*.

DNA replication, and immunoglobulin class switching. However, they can also be generated as transcriptional by-products that compromise genome integrity.<sup>30</sup> This has been shown for cells from yeast to humans depleted of a number of proteins involved in RNA metabolism, including the THO complex of yeast, *Caenorhabditis elegans*, and human cells,<sup>31-33</sup> the ASF/SF2 splicing factor of chicken and human cells,<sup>34</sup> the yeast and human topoisomerase I,<sup>35,36</sup> the yeast DNA-RNA helicase Sen1/Senataxin,<sup>37</sup> and the yeast non-canonical polyA-polymerase Trf4.<sup>38</sup> In addition, several screenings in yeast and human cells have revealed a number of RNA-processing factors preventing R-loop-mediated genome instability.<sup>39-41</sup> Interestingly, absence of Npl3 leads to genome instability, as *npl3Δ* cells accumulate DSBs and have high levels of recombination.<sup>11</sup> This hyperrecombination is dependent on transcription and partially suppressed by overexpression of RNase H1. Therefore, these results strongly support a model suggesting that an improper mRNP assembly may lead to R-loop accumulation in cells lacking Npl3.

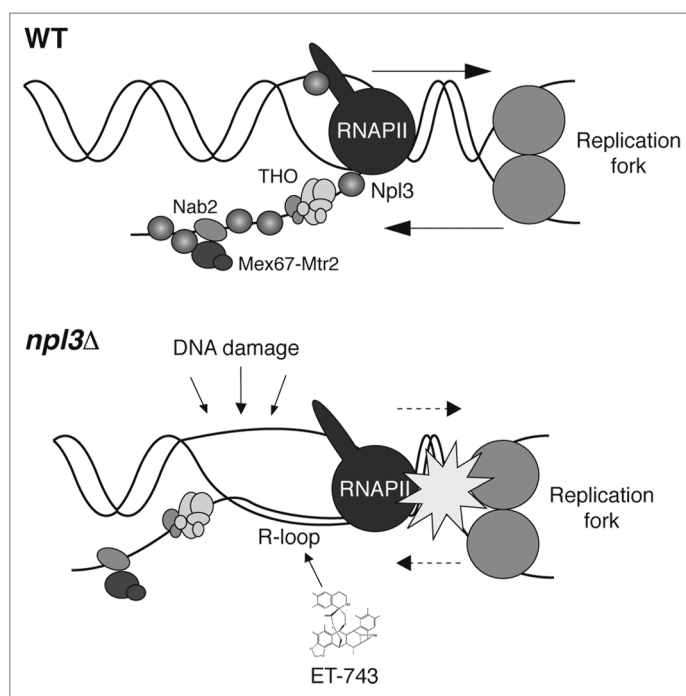
Interestingly, the hyperrecombination phenotype of *npl3Δ* cells, as well as their sensitivity to genotoxic agents and lethality at 37 °C, is suppressed by overexpression of several hnRNP genes.<sup>11</sup> This includes the THO-associated protein Sub2 and the polyA-binding proteins Nab2 and Tho1. Complete suppression of hyperrecombination by *SUB2* overexpression may indicate a function of Npl3 related to that of THO/TREX, at early steps of mRNP biogenesis. Despite their different structures (Fig. 1), both Nab2 and Npl3 share functional similarities. They bind poly-A RNA, function as Mex67 adaptors, shuttle between the nucleus and the cytoplasm, and participate in pre-ribosomal subunits export.<sup>8,20,42-44</sup> Whereas Nab2 associates with mRNAs encoding transcription-related proteins, Npl3 preferentially binds to transcripts encoding ribosomal proteins and other highly transcribed mRNAs,<sup>45</sup> suggesting specific functions for Nab2 and Npl3 in alternative mRNP biogenesis pathways. Conversely, overexpression of *NPL3* reduces the hyperrecombination phenotype of the THO mutant *hpr1Δ*, suggesting the existence of partially

overlapping functions of Npl3 and THO. In this sense, it is worth noting that *NPL3* overexpression per se is able to increase recombination in wild-type cells, so that Npl3 cellular levels has to be tightly regulated. However, a contribution to this phenomenon of late transcription termination caused by excess of Npl3 cannot be discarded.<sup>18</sup>

### Coordination of Transcription, mRNA Export, and Replication to Prevent Genome Instability

Transcription may become an obstacle for replication progression, so that a tight coordination is required for the RF to traverse transcribed DNA sequences.<sup>46</sup> In addition, transcription may occur in close contact to the NPC, according to the “gene gating” hypothesis, thanks to the participation of a number of mRNA export factors and components of the NPC, as shown in yeast, worms, flies, and mammals.<sup>47,48</sup> In this sense, a mechanism

has been proposed by which the DNA damage checkpoint releases transcribed genes from the NPCs to facilitate RF progression.<sup>49</sup> A non-proficient transcription and/or mRNA export may lead to replication impairment, as shown by the genome-wide analysis of the replicative Rrm3 helicase distribution in yeast. As Rrm3 is required for the RF to pass through obstacles,<sup>50</sup> its accumulation at specific genomic regions has been used to identify RF pauses or stalls. Indeed, Rrm3 binding sites highly correlate with transcribed areas.<sup>51</sup> Deletion of *NPL3* increases the genome-wide accumulation of Rrm3 to the highest transcribed genes.<sup>11</sup> Interestingly, Rrm3 accumulation is suppressed by RNase H1 overexpression, suggesting that R loops constitute an obstacle to RF progression in *npl3Δ* cells. Mutants of the THO component Hpr1 also show R-loop-mediated Rrm3 accumulation at highly transcribed genes,<sup>24</sup> suggesting that co-transcriptional R-loops may lead to genome instability by interfering with RF progression.



**Figure 2.** Model illustrating the role of Npl3 coupling mRNP biogenesis and export. In wild-type cells (top), the mRNA is properly assembled into an export-competent mRNP with the collaboration of RNA-binding factors such as Npl3, THO/TREX, Nab2, and Mex67-Mtr2. This facilitates RF progression through transcribed DNA regions. However, in *npl3Δ* cells (bottom) the mRNA packaging is defective, and the nascent mRNA may hybridize with the transcribed DNA strand, leading to R loops that favor the accessibility of DNA damaging agents to the non-transcribed single-stranded DNA and that constitute an obstacle for RF progression. Antitumor drug trabectedin (ET-743) might act similarly to an R loop.

Taking into account the reported effects of Npl3 on transcription, replication, and genome integrity, our current view is that Npl3 is a key player preventing conflicts between these processes. Npl3 stimulates transcription elongation and is highly abundant at the nascent mRNPs, helping prevent transcription–replication conflicts and R-loop accumulation (Fig. 2). In the absence of Npl3, transcription elongation is slower and the mRNAs would not be properly assembled into export-competent mRNPs, enhancing the probability of R-loop accumulation and transcription-associated genome instability.

### A Possible Role of Npl3 in the DNA Damage Response

Although a direct role of RBPs in DNA repair and the DDR has not yet been established, several reports have revealed new functions of specific RBPs in DNA repair in higher eukaryotes.<sup>52</sup> In yeast, different mRNA export mutants, including THO mutants *hpr1Δ* and *tho2Δ* and THSC/TREX-2 mutant *thp1Δ*, show increased sensitivity to genotoxic agents, such as 4NQO, UV, or MMS.<sup>53</sup> Interestingly, the *npl3Δ* mutant is sensitive to MMS, HU, phleomycin, and UV, agents causing DNA lesions that can result in stalled RFs and/or DSBs, indicating a role of Npl3 preventing DNA damage accumulation.<sup>11</sup> Importantly, simultaneous deletion of *NPL3* and *RAD52* or *YKU80*, 2 key components of the homologous recombination (HR) and non-homologous end-joining (NHEJ) pathways of DSB repair, respectively, induces increased sensitivity to MMS, suggesting a connection between Npl3 and DSB repair. Although *npl3Δ* cells proficiently repair DSBs by sister-chromatid recombination, the main HR mechanism, they show a mild defect in the repair by NHEJ. This suggests the possibility that Npl3 had a role in DNA repair consistent with that reported for other RBPs, as in the case of the human p54nrb/NONO, which binds to DSBs sites and favors their repair by NHEJ over HR.<sup>54</sup> On the other hand, sensitivity of *npl3Δ* cells to UV may imply a connection of Npl3 with the nucleotide excision repair (NER) pathway, which repairs bulky adducts

in the DNA, such as those induced by UV. Consistently, several mRNA export mutants (*hpr1Δ*, *tho2Δ*, *sub2Δ*, and *thp1Δ*) have been involved in transcription-coupled NER (TC-NER), the RNAPII-dependent NER sub-pathway.<sup>55</sup> However, this phenotype may also be the consequence of an increase in the collisions between transcription and replication promoted by UV lesions occurring during the S phase, which would lead to DNA breaks that would demand DSB repair functions for their repair and RF restart.

DNA repair genes are not downregulated in *npl3Δ* cells,<sup>11</sup> so that it seems clear that Npl3 does not specifically control the expression of DNA repair genes that could explain their repair phenotypes. Interestingly, however, Npl3 was identified in a proteomic screening for Mec1/Tel1- and Rad53-dependent phosphorylation sites, together with components of the nuclear basket of the NPC.<sup>56</sup> Therefore, it is possible that Npl3 also plays a role in the DDR. Extensive work will be required to understand the molecular mechanisms underlying the connection of yeast RBPs and DNA repair.

One important observation in our study is the hypersensitivity of *npl3Δ* cells to the antitumor drug trabectedin. The mechanism of action of this drug has been proposed to be dependent on the NER machinery both in fission yeast and humans.<sup>57,58</sup> Binding of trabectedin to the DNA would form an adduct recognized by the NER machinery, leading to the stabilization of a complex with the fission yeast/human nuclease Rad13/XPG (Rad2 in budding yeast). The Rad13–DNA–trabectedin ternary complex would block the repair by NER, leading to DSBs and DNA damage checkpoint activation.<sup>57,59</sup> A similar situation was reported for the *rad3–102* mutant in budding yeast, which encodes a defective Rad3/XPD allele that blocks NER repair in a post-incision step and requires HR for RF restart.<sup>60</sup> Consequently, *npl3Δ* cells treated with trabectedin could over-accumulate DSBs, mimicking the effect of MMS or phleomycin.

An additional, but a non-excluding possibility, to explain trabectedin hypersensitivity could be related to the fact that trabectedin binding to the DNA generates

an R-loop-like structure.<sup>61</sup> According to this, Npl3 could be required to prevent or solve this sort of structure, thus explaining the R-loop-mediated genome instability and replication impairment of *npl3Δ* cells. Given the importance of the Npl3 resistance to trabectedin anti-tumor drug, and that genome instability is a common hallmark of cancer cells,<sup>62,63</sup> these observations open the possibility of exploring the use of specific RBPs as new targets for anti-cancer drug designs.

### Concluding Remarks

Transcription-associated genome instability is a common hallmark of mRNA processing defects from yeast to human cells. Depletion of proteins involved in mRNP biogenesis and processing leads to an increase in DNA breaks in a transcription-dependent manner, in part due to R-loop formation, and as a consequence of replication impairment. Finding that the yeast SR-like protein Npl3, one of the most abundant RNA-binding proteins in yeast, prevents genome instability and R-loop formation as the major trigger of transcription-associated instability provides strong evidence of the key importance of proper mRNA biogenesis and assembly in preventing the nascent RNA from interacting with the template DNA and from compromising genome integrity. Thus, Npl3 is a multifunctional protein acting from transcription to mRNA processing and export with a function in preventing genome instability. Interestingly, it seems to have additional roles in DNA repair, as is the case of other RBPs from mammalian cells, although this is still poorly understood. Thus, RBPs constitute a conserved group of nuclear factors that safeguard genome integrity in many different ways. Defining the specific functions of Npl3 and other RBPs in genome integrity, as well as their involvement in DNA repair, the DDR, and cancer is important to understand the role of RNA and RNA-binding factors in genome dynamics and in the origin of cancer.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.



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