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EXTRA VIEW



Ddx19 links mRNA nuclear export with progression of transcription and replication and suppresses genomic instability upon DNA damage in proliferating cells

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ABSTRACT

The DEAD-box Helicase 19 (Ddx19) gene codes for an RNA helicase involved in both mRNA (mRNA) export from the nucleus into the cytoplasm and in mRNA translation. In unperturbed cells, Ddx19 localizes in the cytoplasm and at the cytoplasmic face of the nuclear pore. Here we review recent findings related to an additional Ddx19 function in the nucleus in resolving RNA:DNA hybrids (R-loops) generated during collision between transcription and replication, and upon DNA damage. Activation of a DNA damage response pathway dependent upon the ATR kinase, a major regulator of replication fork progression, stimulates translocation of the Ddx19 protein from the cytoplasm into the nucleus. Only nuclear Ddx19 is competent to resolve R-loops, and down regulation of Ddx19 expression induces DNA double strand breaks only in proliferating cells. Overall these observations put forward Ddx19 as an important novel mediator of the crosstalk between transcription and replication.

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Introduction

Transcription and replication are two fundamental cellular processes that occur within the nucleus. The nascent mRNA generated during transcription is processed, spliced and exported to the cytoplasm, a series of actions that are coupled to ongoing transcript synthesis. Whereas these processes can be temporally separated, as in quiescent cells or during early embryogenesis, in proliferating cells they occur simultaneously. This situation certainly requires both a functional and structural organization of the genome within the nucleus, so to avoid interference between these events. Indeed, early experiments in mammalian cells provided evidence for the presence of replication and transcription factories as independent structural nuclear compartments.¹ Despite this compartmentalization, these two processes are very likely to cross paths on their common DNA template in the S-phase of the cell cycle. Conflicts thereby are bound to occur, particularly in situations in which transcription and replication travel in opposite

directions (head-on collisions), upon unscheduled activation of DNA synthesis driven by aberrant activation of an oncogene in differentiated cells, or upon stalling of either machineries in presence of natural obstacles or DNA damage. When these conflicts arise, aberrant RNA:DNA hybrids accumulate on the transcription unit, also known as R-loops, where the nascent mRNA anneals back to its DNA strand displacing a single stranded DNA portion. These structures exert physiologic functions in immunoglobulin class switch recombination, mitochondrial replication, gene expression regulation, influence epigenetic environment, and even in DNA repair. Their persistence however can generate a dangerous load of DNA damage such as double strand breaks (DSBs) that threaten the integrity of the genome (for review see ref. 2). Two reports have previously implicated the DNA damage response pathway in the coordination of these processes by recruitment of the Senataxin helicase³ or to remove RNA bound proteins from damaged DNA.⁴ In a search for new DNA

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damage responsive genes, using *in vitro* protein extracts derived from fertilized eggs of the amphibian *Xenopus laevis*, we have identified the RNA helicase Ddx19 and revealed a novel role for this enzyme in R-loop metabolism, in resolving conflicts between replication and transcription in proliferating cells.

Ddx19 functions in mRNA export and translation from yeast to humans

Ddx19 is an essential gene conserved in eukaryotes belonging to the superfamily-2 of DEAD box helicase whose product displays both ATPase and RNA unwinding activity.^{5,6} Ddx19 was initially identified as a putative RNA helicase with unknown function in a screen for DEAD-box protein-encoding genes in yeast, and named Dbp5, for DEAD-box protein 5,⁷ also known as Rat 8 for Ribonucleic acid-trafficking protein 8. Another genetic screen for temperature-sensitive mutants with mRNA export defects in yeast, and in parallel an independent study in mammalian cells, attributed a function to Ddx19 in mRNA export at the cytoplasmic face of the nucleopore.^{5,6,8} Ddx19 localizes primarily in the cytoplasm with a fraction concentrated at the Nuclear Pore Complex (NPC) at the cytoplasmic fibrils, *via* an interaction with Nup159/Rat7 in yeast and its mammalian counterpart Nup214/CAN at the N-terminal domain (NTD) of the nucleoporin.^{5,8,9} It is notwithstanding that, though Δ NTD-*nup159* mutants lost the ability to bind Dbp5, they had no effect on its helicase/ATPase activity,^{5,9} indicating that binding of Dbp5 to Nup159 is not the rate-limiting step in mRNA export which depends upon the Gle1 protein (see below).

As a member of the DEAD-box helicases family, Ddx19 also possesses the DEAD core helicase domain and contains 13 characteristic sequence motifs. It is also characterized by the two Rec A-like domains that contribute to binding of RNA, ATP, and its two main interaction partners Nup214/CAN and Gle1 (see below). The ATP-bound Ddx19 preferentially binds to RNA that dissociates upon ATP hydrolysis, while the ADP-Ddx19 is recycled for binding other RNA molecules.

Unlike conventional helicases, DEAD-box helicases have no specific requirement for a defined polarity (though some exhibit preferences) and show low processivity (25 up to 40 bp). *In vitro* Ddx19 displays both RNA-dependent ATPase and ATP-dependent RNA

unwinding activities that are intrinsically low,^{6,8} but are stimulated upon interaction with Gle1.^{10,11} Gle1 is an export factor whose binding site to Nup42 (hCG1 in mammals) at the cytoplasmic face is juxtaposed to that for Dbp5.¹² Indeed Gle1, with its cofactor inositol hexakisphosphate IP6, were identified as the ATPase activator of Dbp5, and interaction of Dbp5 with Gle1-IP6 was shown to be the limiting step for mRNA export.^{10,11} Placed at the cytoplasmic filaments of the NPC, Dbp5 is perfectly positioned to trigger the remodeling of the emerging cytoplasmic mRNPs and initiate the replacement of export factors, primarily the export factor Mex67 (NXF1/TAP in mammalian cells) that otherwise would be retained on mRNA, particularly on the nuclear rim when the exosome was impaired.¹³

A cytoplasmic function for Ddx19 was proposed when in yeast Dbp5 was found to associate with polyribosomes and to interact with eukaryotic release factors eRF1 and eRF3, suggesting a role in translation. Since a helicase mutant form of Dbp5 displayed increased read-through activity, the authors proposed a model for efficient translation termination where upon reaching a stop codon and binding of eRF1, Dbp5 would remodel mRNA/protein complexes. This allows proper positioning of eRF1, followed by release of Dbp5 and recruitment of eRF3 for subsequent downstream events.¹⁴

A putative Ddx19 role during transcription?

Immuno-electron microscopy observation of polytene chromosomes of the salivary gland in the larva of *C. tentans*, revealed an association of Dbp5 with the mRNP of the Balbiani ring within the nucleus that persisted during mRNA export and translation. Hence the authors proposed a general unknown function for Dbp5 in transcription.¹⁵ However, their conclusions were challenged by more recent studies using light sheet fluorescence microscopy aimed at following the trajectory of the export of single RNA molecules in living *C. tentans* salivary gland cells. In this live cell imaging setup, nuclear probing of Dbp5 was minimal, whereas cytoplasmic probing, from the cytoplasm to the nuclear envelope then back to the cytoplasm, was prevailing.¹⁶ In *S. cerevisiae*, a genetic and physical interactions between Dbp5 and the TFIIF transcription factor complex having dual roles in transcription initiation and DNA repair was reported.¹⁷ The partial

suppression of export defects observed in the temperature-sensitive mutant strain *rat8-2* by mutations in transcription initiation but not elongation factors, led the authors to propose an early unknown role for Dbp5 in transcription initiation. However, no interaction of Dbp5 with chromatin was detectable in yeast, and furthermore Ddx19 was not detected on nascent transcripts in mammalian cells; hence it remained unclear the functional relevance of such a controversial nuclear localization.

Ddx19 as a DNA damage-responsive gene

We identified Ddx19 during an *in vitro* search for DNA damage responsive genes in *Xenopus* egg extracts. We observed that *Xenopus* Ddx19 translocates into nuclei reconstituted in extracts and damaged by UV-C irradiation. At the beginning this finding was somehow puzzling since in this system transcription is naturally turned off. What could be a role for the nuclear Ddx19 then? The answer to this question came upon extensive characterization of Ddx19 function in mammalian cells, in which we could observe that GFP-tagged Ddx19 translocates into the nucleus of live cells upon UV-irradiation, in an ATR-dependent fashion. In fact, inhibition of ATR, and not ATM, blocked Ddx19 nuclear translocation upon UV damage. Indeed Ddx19 was previously identified as substrate of the Chk1 kinase, a main ATR target.¹⁸ Hence, because in *Xenopus* egg extract ATR is activated upon UV irradiation, we believe that its nuclear translocation is a default mechanism driven by ATR.

A novel nuclear function for Ddx19 as an RNA:DNA hybrids resolving enzyme

Down regulation of Ddx19 in mammalian cells was previously reported to increase the levels of the phosphorylated form of the histone variant H2AX (γ H2AX),¹⁹ a marker of DNA damage and replication stress. We confirmed this original observation and in addition found that Ddx19 down regulation was selectively activating the ATM kinase pathway that responds primarily to DSBs, leading to p53 phosphorylation. The presence of DSBs was directly confirmed by pulse field gel electrophoresis and accumulation of nuclear foci of the 53BP1 protein, a specific marker of DSBs. Because Ddx19 functions at the cytoplasmic face of the NPC, we were expecting that its down

regulation would generate DNA damage limited to the chromatin space close to the nuclear periphery, as we observed following down regulation of the nuclear export factor Nxf1. Surprisingly, Ddx19 down regulation generated both γ H2AX and 53BP1 foci scattered throughout the nucleus. This first observation prompted us to look for an additional function of Ddx19 in the nucleus. Because we also observed that Ddx19 down regulation slows down DNA synthesis and generates proliferation-dependent DNA damage, we reasoned that Ddx19 might be implicated in resolving conflicts between replication and transcription. Consistent with this possibility, we did not observe γ H2AX in replicating, transcription-incompetent nuclei assembled in *Xenopus* egg extracts depleted of Ddx19, while this was observed when the extracts were artificially set for transcription. In addition, the slow down of DNA synthesis observed in mammalian cells upon Ddx19 depletion was rescued when cells were treated with the RNaseH1 enzyme that degrades the RNA moiety in RNA:DNA hybrids, suggesting that Ddx19 may be involved in R-loop metabolism. We first confirmed this possibility *in vitro* by demonstrating that recombinant human Ddx19 is able to unwind these structures. Then, we were also able to detect R-loops directly *in vivo* in mammalian cells by using the S9.6 antibody that recognizes RNA:DNA hybrids in both immunofluorescence and immunoprecipitation experiments (DRIP), or by using a fluorescently-tagged mutant of RNaseH that remains bound to the R-loop.⁴ We could observe a large number of RNaseH-sensitive R-loops scattered throughout the nucleus in cells depleted of Ddx19, while upon Nxf1 depletion R-loops were mainly located around the nuclear periphery. But can DNA damage and R-loops accumulation observed upon Ddx19 knock-down be explained as an indirect effect on transcription? Down regulation of several mRNA binding factors has been previously reported to induce phenotypes similar to Ddx19 depletion,¹⁹⁻²¹ which is expected since inhibition of mRNP maturation in the nucleus will slow down transcription. However, because Ddx19 is at the end of the mRNA journey from the transcription unit to the export gate, it is unlikely that its inhibition would directly lead to a slowdown of transcription. Consistent with this possibility, we did not observe R-loop formation in cells depleted of the Ddx19 co-factor in mRNA export Gle1. Intriguingly however, Gle1 knockdown also

induced γ H2AX, though to a lower extent than Ddx19 knockdown. Why then inhibition of mRNA export at the NPC generates DNA damage and/or replication stress? One possibility may be that inhibition of export of mRNP particles would limit the recycling of mRNA processing proteins and therefore indirectly slow down transcription, although other scenarios may also be possible. Notwithstanding, Ddx19 knockdown generates a higher level of DNA damage compared with Nxf1 or Gle1 knockdown, supporting an additional export-independent role of the former.

The RNA:DNA hybrid resolving activity of Ddx19 is Chk1-dependent

To identify separation of functions domains in Ddx19, we analyzed the ability of different Ddx19 mutants to resolve R-loops that accumulate in cells treated with the chemotherapeutic-relevant agent camptothecin (CPT), a DNA Topoisomerase I inhibitor. We observed that both the helicase and the RNA binding domain in Ddx19 were required to remove CPT-induced R-loops. Importantly, these were still persistent in cells overexpressing Nxf1, despite its nuclear localization, showing that CPT-induced R-loops cannot be reduced by increasing mRNA export. Taken together these observations suggest that R-loop accumulation in cells depleted of Ddx19 cannot be solely explained as a result of a defect in mRNA export. This conclusion is also supported by the observation that a Ddx19 mRNA export-defective mutant could resolve these structures as efficiently as the wild-type. In line with this finding, expression of RNaseH1 only partially suppressed the global DNA damage generated by Ddx19 knockdown. Because RNaseH1 resolves R-loops, the residual DNA damage (about 50%) observed in these cells is probably arising from mRNA export defects, as observed in cells depleted of either Gle1 or Nxf1. We also generated non-phosphorylatable and phosphomimetic Ddx19 mutants in the Chk1 phosphorylation site, previously mapped on Serine 93.¹⁸ Strikingly, we could observe that Ddx19 refractory to Chk1 phosphorylation never goes into the nucleus and does not resolve R-loops. On the contrary, the Ddx19 phosphomimetic mutant was always nuclear and was also very effective in resolving R-loops. Altogether, these observations show a dual, bi-functional trajectory of Ddx19 in the cell: a shuttle from the NPC toward the cytoplasm for mRNA

export and translation termination, and an opposite shuttle from the NPC toward the interior of the nucleus orchestrated by the ATR-Chk1 pathway to resolve deleterious R-loops.

Our observations propose Ddx19 as a novel enzyme required to resolve R-loops in mammalian cells, directed by the ATR checkpoint pathway (Fig. 1). By sensing slowing down of replication forks, generated by natural obstacles (such as difficult to replicate DNA and/or chromatin structures), conflicts with transcription units, or by DNA damage, ATR leads to Chk1-dependent Ddx19 phosphorylation. It is likely that this post-translational modification alters the localization of Ddx19, since we have observed that the Chk1-phosphomimetic mutant of Ddx19 does not interact with the Nup214 nucleoporine. Once in the

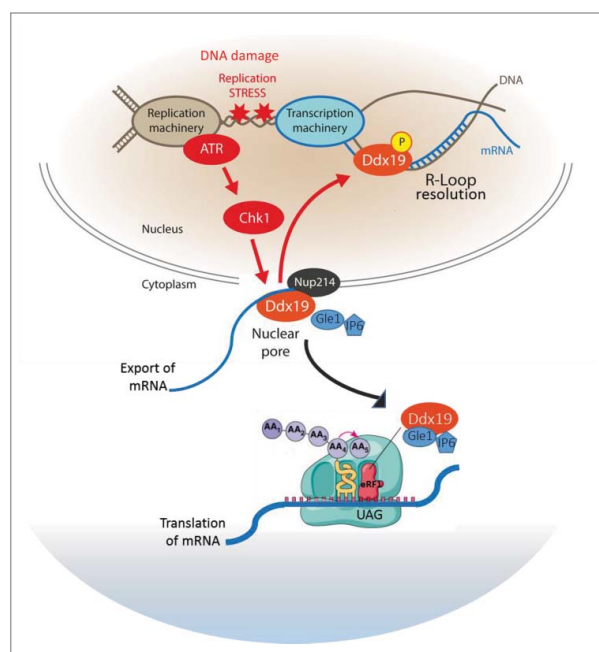


Figure 1. Ddx19: a multifaceted helicase on the spot. The DEAD-Box helicase Ddx19 is a multifunctional protein, executing diverse functions that are well dedicated to its different cellular localizations. Tethered to the nucleoporine Nup214 at the cytoplasmic fibrils of the nuclear pore complex, Ddx19 remodels arriving mRNPs at the pore to export them into the cytoplasm, stimulated by Gle1-IP6 factors. The cytoplasmic pool of Ddx19 is involved in translation termination by remodeling mRNA/proteins to allow proper positioning of the release factor eRF1 when a stop codon is reached. Very recently, we attributed a new nuclear role for Ddx19 in resolving aberrant RNA:DNA hybrids known as R-loops, which arise upon conflicts between replication and transcription. These conflicts create replication stress that is sensed by the ATR-Chk1 checkpoint pathway. The Chk1 kinase subsequently phosphorylates Ddx19 to trigger its translocation from its peripheral localization toward the chromatin to resolve these harmful structures. (Adapted from Hodroj et al., 2017).

nucleus, Ddx19 may resolve R-loops alone or in combination with other factors that remain to be identified. Nuclear localization of Ddx19 increases its stability, and as we have observed, this depends upon ATR. Hence, when the ATR signal is dampened, Ddx19 may be dephosphorylated by an unknown phosphatase and then probably destabilized by proteasomal degradation. Then, newly synthesized Ddx19 will remain in the cytoplasm and be anchored to the nucleopore because of low ATR activity. The findings we have reported in mammalian cells fit into a described previously “gene gating” model in yeast, proposing that conflicts between transcription and replication activate ATR leading to phosphorylation of Chk1 substrates at the nuclear pore to transiently inhibit nuclear export and facilitate the passage of the replication fork through a transcription unit. In this situation, the mRNA released from the nucleopore would anneal back to its template DNA generating an R-loop.²² Ddx19 would be then recruited to remove the R-loop and make the mRNA again available for export. However evidence for a “gene gating” mechanism in mammalian cells is missing. Our findings show that the DNA damage response not only links regulation of transcription and replication with nuclear export, but also promotes recruitment of specific factors required to remove potentially harmful structures on the DNA.

Ddx: A new paradigm in R loop metabolism

Cells appear to have evolved several enzymes to deal with R-loops. RNaseH degrades the RNA moiety in this structure with no sequence specificity,²³ The helicase Senataxin (sen1 in yeast) plays a crucial role in facilitating transcription termination and its absence causes R-loop accumulation²⁴ as well as transcript read-through due to problems in transcription termination.²⁵ Also, an essential role for Senataxin during spermatogenesis, in meiotic recombination was reported.²⁶ followed more recently by the FANCM DNA translocase²⁷ and the mRNA processing helicase Aquarius.²⁸ Interestingly, two other DEAD-Box helicases have also very recently been implicated in R-loop metabolism: Ddx1 and Ddx23. Ddx1 has been shown to form ionizing radiation-induced foci that are sensitive to treatment with RNaseH or transcription inhibitors, and the authors could show that RNA:DNA hybrids accumulate around a DSB when Ddx1

is depleted. These results led to the proposal that Ddx1 is recruited to resolve RNA:DNA hybrids at DSBs to facilitate repair and homologous recombination.²⁹ In an independent study, increased DNA damage and R-loop formation was also observed in Ddx23 depleted cells.³⁰ The authors demonstrated by chromatin immunoprecipitation experiments that this helicase accumulates on R-loop-containing loci, driven by pausing of transcription complexes, upon its phosphorylation by the serine/arginine protein kinase 2 (SRPK2).³⁰ Hence, these new findings now change our perception of these ubiquitous multifunctional enzymes that make up the largest family of RNA helicases found across all forms of life. Previous knowledge has implicated them in cancer and differentiation. Now, they are in the front line for maintenance of genome stability. It would be then important in the future to understand the choreography of all these actors on the genome landscape and determine which endogenous and exogenous cues recruit them.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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