

The Ro Autoantigen Binds Misfolded U2 Small Nuclear RNAs and Assists Mammalian Cell Survival after UV Irradiation

Xinguo Chen,¹ James D. Smith,¹
Hong Shi,¹ Derek D. Yang,²
Richard A. Flavell,² and Sandra L. Wolin^{1,*}

¹Department of Cell Biology and
Howard Hughes Medical Institute
Yale University School of Medicine
New Haven, Connecticut 06536

²Section of Immunobiology and
Howard Hughes Medical Institute
Yale University School of Medicine
New Haven, Connecticut 06510

Summary

The Ro 60 kDa autoantigen, an RNA binding protein, is a major target of the immune response in patients with systemic lupus erythematosus. As mice lacking Ro develop a lupus-like syndrome, Ro may be important for preventing autoimmunity [1]. However, the cellular function of Ro, which binds small cytoplasmic RNAs of unknown function called Y RNAs, has been enigmatic. Ro has been proposed to function in 5S rRNA quality control based on experiments in *Xenopus laevis* oocytes [2], and a Ro ortholog enhances survival of the eubacterium *Deinococcus radiodurans* after ultraviolet irradiation [3]. To test the general importance of these two observations for Ro function, we investigated the role of Ro in mammalian cells. We report that, in mouse embryonic stem (ES) cells, Ro binds variant spliceosomal U2 snRNAs. Expression of mouse U2 snRNAs in *Xenopus* oocytes reveals that binding occurs in nuclei and appears to involve recognition of misfolded RNA. Moreover, mouse ES cells lacking Ro exhibit decreased survival after ultraviolet irradiation. In irradiated cells, both Ro and a Y RNA accumulate in nuclei. We propose that Ro plays a general role in small RNA quality control and that this function is important for cell survival after ultraviolet irradiation.

Results and Discussion

Ro Associates with a Collection of Largely Variant U2 RNAs in ES Cells

Earlier studies suggested two roles for Ro. One is in 5S rRNA quality control, because in *Xenopus* oocytes, Ro binds variant pre-5S rRNAs that are misfolded, inefficiently processed, and eventually degraded [2, 4]. The other role is as part of a cell survival mechanism after UV irradiation, as seen for a Ro ortholog in the radiation-resistant eubacterium *Deinococcus radiodurans* [3]. Are these general functions of Ro? To address this question, we investigated Ro function in mammalian cells.

First, we created mouse embryonic stem (ES) cells lacking Ro (see Supplemental Experimental Procedures).

Western blotting confirmed that Ro was undetectable in *Ro*^{-/-} cells (Figure 1A). Northern analyses revealed that the two mouse Y RNAs mY1 and mY3 were drastically reduced (Figure 1B), consistent with reports that Ro stabilizes these RNAs [1, 5]. Immunoprecipitation with an antibody against the La protein, which binds nascent RNA polymerase III transcripts [6], revealed that the few Y RNAs in *Ro*^{-/-} cells are bound by La (data not shown). To verify that any phenotypes were due to loss of Ro, we also generated stable lines in which Ro was expressed in *Ro*^{-/-} cells under control of the mouse phosphoglycerate kinase promoter (*PGK-Ro*). Western blotting identified several lines that expressed Ro at slightly less than wild-type levels (Figure 1A). Northern blotting revealed that Y RNA levels increased in *PGK-Ro* cells (Figure 1B).

To examine the RNAs bound by Ro, wild-type, *Ro*^{-/-}, and *PGK-Ro* cell lysates were subjected to immunoprecipitation with an antibody against mouse Ro. RNAs within immunoprecipitates were labeled with [³²P]pCp. On light exposures, only Y RNAs were detected in anti-Ro immunoprecipitates. On long exposures, a band was visible that comigrated with the U2 snRNA (Figure 1C, asterisk). The band was absent from the *Ro*^{-/-} immunoprecipitate, indicating it was associated with Ro. Low levels of 5S and 5.8S rRNAs were nonspecific contaminants, as they were present in *Ro*^{-/-} immunoprecipitates (Figure 1C).

To identify the RNA, we synthesized and sequenced cDNAs. The band consisted of a population of largely variant U2 snRNAs (Figure 1D). Of 38 cDNAs, 26 (68%) contained changes from wild-type U2 snRNA (see Table S1). Variants of the spliceosomal U1, U2, U4, and U5 snRNAs are expressed in organisms from plants to humans [7–9]. To examine the entire U2 snRNA population in ES cells, we prepared cDNA by using total U2 RNA as template. Approximately half the U2 snRNAs (20/38; 53%) contained changes from the wild-type snRNA. However, comparison of variant U2 snRNAs in anti-Ro immunoprecipitates with those in total RNA revealed that the distribution of changes between the two populations differed (Table S1). At least some of the variant U2 snRNAs appear genome encoded [10] (Table S1). If mouse U2 snRNAs, like human, are encoded in tandem repeats, the fraction encoded in the genome is an underestimate, as these repeats are underrepresented in genome sequencing [11]. Also, some variants could represent transcriptional errors or RNA editing events.

Binding of *Xenopus* Ro to Variant U2 snRNAs Occurs in Nuclei and Requires Specific Nucleotide Changes

To test whether Ro associates with wild-type and/or variant U2 snRNAs, we constructed U2 genes that encoded four variant U2 snRNAs in our immunoprecipitates. We chose three variants found multiple times and the Sm site mutant T100C (variants 1–4, Figures 1D and

*Correspondence: sandra.wolin@yale.edu

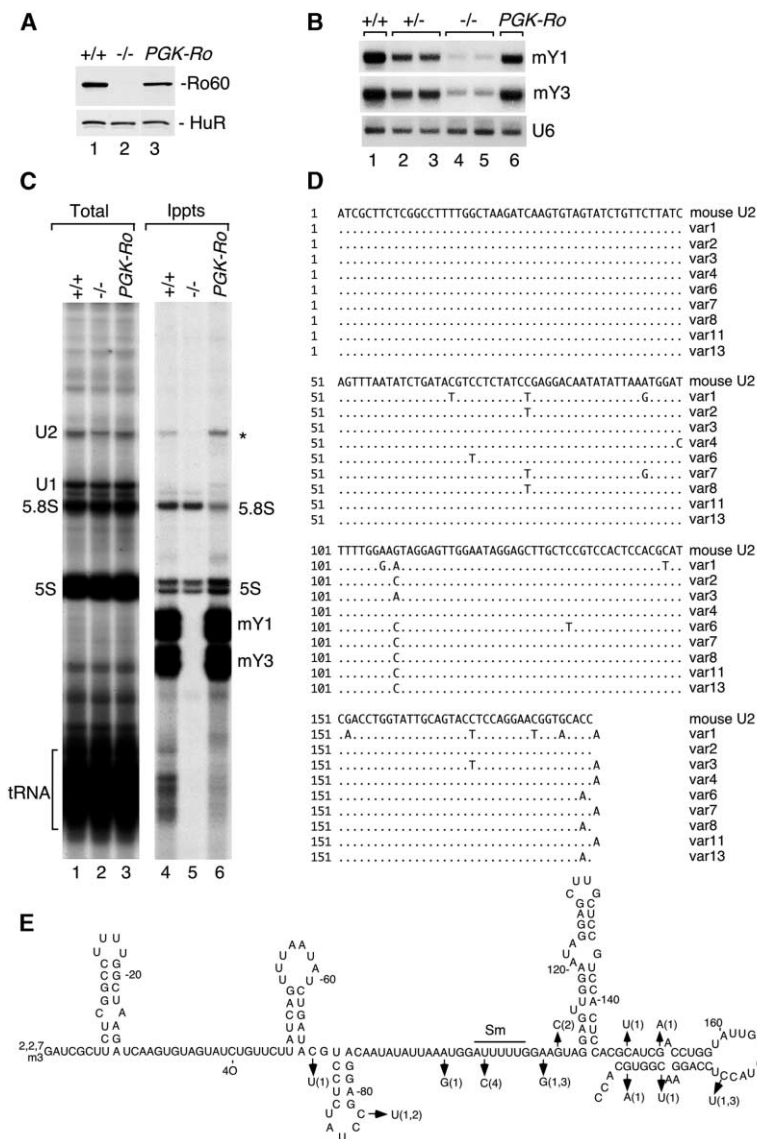


Figure 1. Ro Associates with a Collection of Largely Variant U2 snRNAs

(A) Lysates of wild-type, *Ro*^{-/-}, and *PGK-Ro* cells were subjected to Western blotting to detect Ro. The blot was reprobed to detect HuR.

(B) RNA from the cell lines was subjected to Northern analysis to detect the two mouse Y RNAs. The filter was reprobed to detect U6 snRNA.

(C) Lysates from wild-type, *Ro*^{-/-}, and *PGK-Ro* cells were subjected to immunoprecipitation with anti-Ro antibodies. RNAs in immunoprecipitates (lanes 4–6) and 5% of lysates (lanes 1–3) were extracted and labeled at the 3' end. Asterisk, a mixture of largely variant U2 snRNAs. The levels of variant U2 appear higher in *PGK-Ro* cells (lane 6) than in wild-type cells (lane 4) because more *PGK-Ro* cells were used for the extract.

(D) The sequences of nine U2 cDNA clones from U2 snRNA in anti-Ro immunoprecipitates are compared to wild-type U2. Several clones contain 3' adenosine. Adenosine-containing clones were also identified in total U2 RNA and may represent U2 RNA that undergoes posttranscriptional adenosine addition [20].

(E) The secondary structure of mouse U2 RNA is shown. Changes in variants 1–4 are indicated by parentheses. The Sm site is indicated.

1E). We injected the genes into *Xenopus* oocytes and examined the association of *Xenopus* Ro with the transcripts. We chose oocytes because by labeling transcripts we could avoid introducing additional changes into the RNAs to distinguish them from endogenous snRNAs. Also, U snRNP biogenesis involves nuclear export of 3' extended U RNAs, cytoplasmic assembly and processing, and nuclear reimport [12]. As oocytes can be separated into nuclei and cytoplasm without nuclear leakage, they are useful for examining this pathway.

Each mouse U2 gene was injected into oocytes with [α -³²P]GTP. As a positive control, we coinjected the human Y3 gene. Two forms of U2 RNA were synthesized (Figures 2A and 2B). The larger transcript corresponds to 3' extended pre-U2 [13, 14]. In addition to U2 and hY3 RNAs, transcripts from endogenous 5S rRNA and tRNA genes were visible. Immunoprecipitations revealed that both precursor and mature forms of two variant U2 RNAs but not wild-type U2 associated with Ro (variants

2 and 4; Figures 2A and 2B). In addition, very low levels of a third variant were detected bound to Ro (Figure 2B, variant 3). Thus, three of four variant U2 RNAs were bound by Ro.

To determine the subcellular location of the Ro/U2 snRNA, we coinjected oocytes with the variant 2 U2 gene, the human Y3 gene, and the *Xenopus* U3 small nucleolar RNA gene. After enucleation, U3 RNA was nuclear, while tRNAs were cytoplasmic (Figure 2C). Immunoprecipitations revealed that, while the Ro/Y RNA was cytoplasmic, the Ro/U2 snRNA was nuclear (Figure 2C). Thus, as observed for variant pre-5S rRNAs [2], Ro association with U2 snRNAs may not require Y RNAs, as they are not detected in nuclear Ro/U2 snRNA complexes.

We examined the sequence requirements for Ro binding. As the Sm site mutant (variant 4) contained only the T100C change, this alteration was sufficient for binding. The other variant bound strongly by Ro (variant 2, Figure 2A) contained two changes, C78T and G109C. We con-

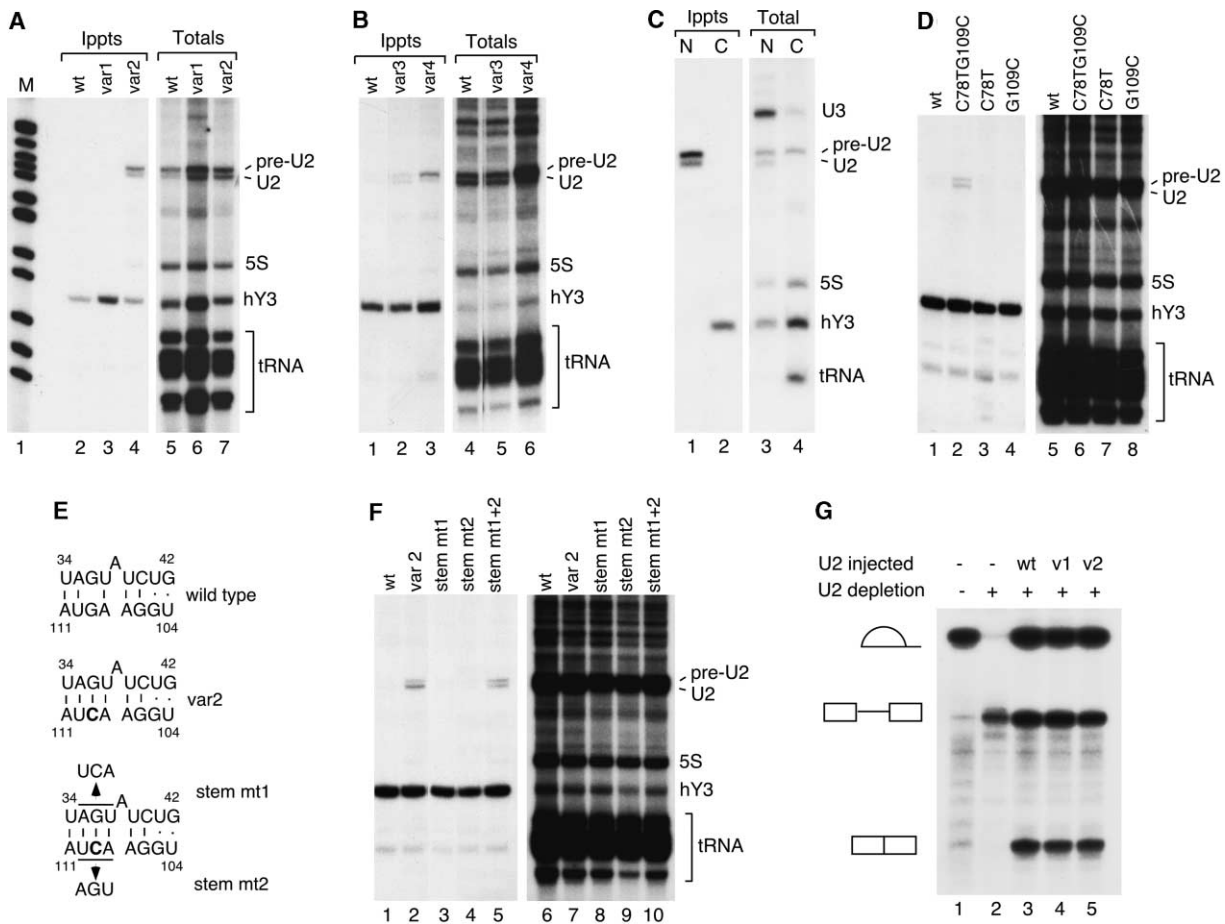


Figure 2. Ro Binding to a Variant U2 RNA Occurs in Nuclei and Requires a Novel Helix

(A) Plasmids containing wild-type and variant U2 genes (var1 and var2) were injected into *Xenopus* oocytes along with the hY3 gene and [α - 32 P]GTP. After 16 hr, lysates were incubated with anti-Ro antibodies (lanes 2–4). Lanes 5–7, total RNA from 5% of lysates. Var1 RNA, which was abundant in ES immunoprecipitates (Table S1), does not bind Ro (lane 3). One possibility is that, as described [21], excess snRNP proteins stockpiled in oocytes allow certain U2 variants that are defective in mammalian cells to assemble into functional snRNPs in oocytes.

(B) Wild-type and variant U2 genes (var3 and var4), the hY3 gene, and [α - 32 P]GTP were injected into oocytes. After 16 hr, oocytes were lysed and incubated with anti-Ro antibodies (lanes 1–3). Lanes 4–6, RNA from 5% of lysates.

(C) The variant 2 U2 gene, the hY3 gene, and a *Xenopus* U3 gene were injected into oocytes with [α - 32 P]GTP. After 16 hr, oocytes were dissected into nuclei (N) and cytoplasms (C) and subjected to immunoprecipitation with anti-Ro antibodies. RNAs in immunoprecipitates (lanes 1 and 2) and totals (lanes 3 and 4; 5% of lysates) are shown.

(D) Variant U2 snRNA genes containing either the C78T or G109C changes, or both changes, were injected into oocytes with [α - 32 P]GTP. After 16 hr, lysates were phenolextracted (lanes 5–8; 5% of lysates) or subjected to immunoprecipitation with anti-Ro antibodies (lanes 1–4).

(E) A potential helix in U2 snRNA is shown. The G109C change in the variant 2 RNA results in increased basepairing within the helix. Also shown are positions of nt changes in the two stem mutants. The parent construct was the variant 2 snRNA.

(F) Plasmids containing wild-type or mutant U2 snRNAs were injected into oocytes along with the hY3 gene and [α - 32 P]GTP. After 16 hr, lysates were phenolextracted (lanes 6–10; 5% of lysates) or subjected to immunoprecipitation with anti-Ro antibodies (lanes 1–5).

(G) Endogenous U2 was depleted by injecting an antisense oligonucleotide into oocytes (lanes 2–5). After 4 hr, oocytes were injected with plasmids containing wild-type and variant U2 genes (lanes 3–5) and incubated 18 hr. Oocytes were then injected with a 32 P-labeled adenovirus splicing substrate and incubated 30 min. Lane 1, no oligonucleotide injection. As noted [15], spliced mRNA is low compared to lariats in uninjected oocytes. In multiple experiments, the unspliced transcript was unstable when splicing was inhibited (lane 2).

structed U2 genes containing each of these changes. U2 transcripts containing only one change were not bound by Ro (Figure 2D). Thus, both changes in the variant 2 RNA are required for Ro association.

Ro Recognition of a Variant U2 snRNA Requires Formation of a Novel Helix

The variant pre-5S rRNAs bound by Ro contain base changes that destabilize two conserved helices, favoring formation of an alternative helix [4]. In contrast,

the changes in the variant U2 snRNAs do not destabilize U2 structure (Figure 1E). However, sequences in U2 that are 3' to the Sm site (nts 103–110) have the potential to basepair with sequences that normally basepair with intron sequences during pre-mRNA splicing (nts 34–42). In wild-type U2, this potential helix is interrupted by a G-G mismatch involving G109 (Figure 2E). In the variant 2 RNA, the G109C change could result in basepairing between G36 and C109, stabilizing the helix (Figure 2E). In this scenario, the second mutation in the variant,

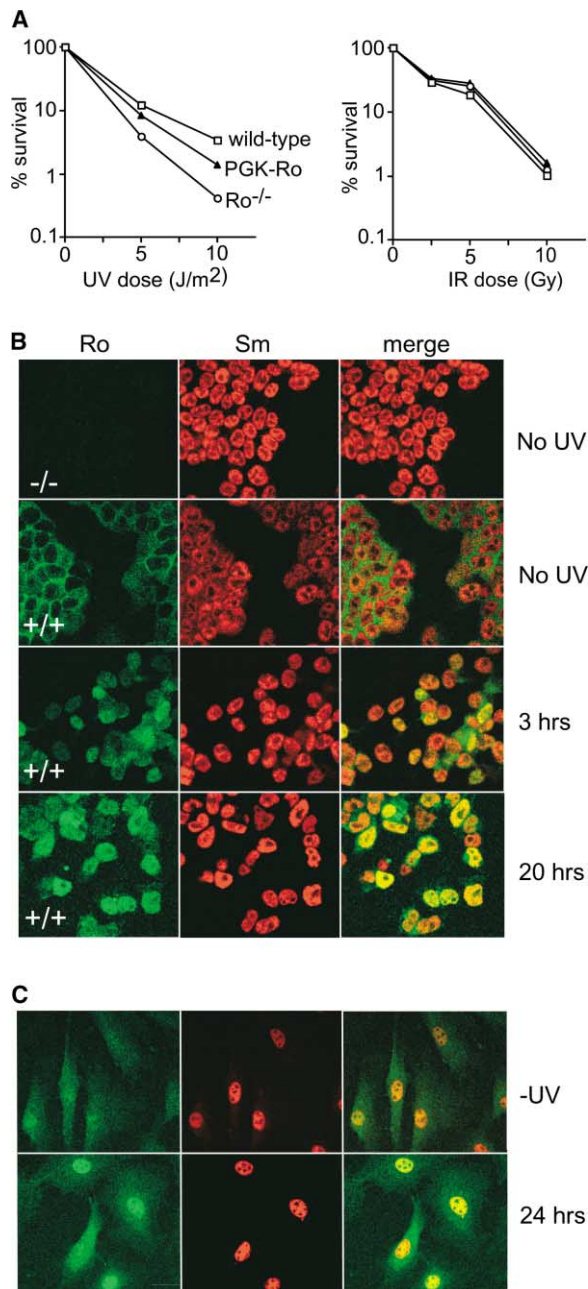


Figure 3. Ro Contributes to Survival after UV and Accumulates in Nuclei after Irradiation

(A) Wild-type, *Ro*^{-/-}, and *PGK-Ro* cells were treated with the indicated doses of UVC (left) or ionizing radiation (right). Cell viability was assessed 72 hr later. Each point represents the mean of three experiments. For each dose, we compared the ratio of the survival of the wild-type versus the *Ro*^{-/-} strain with the Student's *t* test. At both 5 and 10 J/m² UVC, differences in survival rates were significant (*p* < 0.007, using the Bernoulli correction for multiple comparisons). In addition, differences between survival of *PGK-Ro* and *Ro*^{-/-} cells were significant at both UVC doses.

(B) Wild-type and *Ro*^{-/-} ES cells were fixed and stained with rabbit anti-mouse Ro antibodies (left panels) or anti-Sm antibodies (middle panels). Merged images are in right panels. Cells were unirradiated (top two rows) or irradiated with 10 J/m² UVC and allowed to recover for 3 hr (third row) or 20 hr (bottom row).

(C) Wild-type mouse embryonic fibroblasts were unirradiated (top row) or irradiated with 20 J/m² UVC and allowed to recover for 24

hr (bottom). As these cells are less sensitive to UV than ES cells, they were irradiated with higher doses. Similar nuclear accumulation is observed with 10 J/m² UVC. Cells were fixed and stained as in (B).

C78T, may increase formation of the abnormal helix by disrupting protein binding to the intervening stem loop. We constructed mutations in the variant snRNA that disrupted or stabilized the potential helix. Conversion of nts 35–37 to the complementary sequence, which disrupts basepairing in the helix, eliminated Ro binding (stem mt1, Figures 2E and 2F) as did conversion of nts 108–110 to the complementary sequence (stem mt2). When the two stem mutants were combined, restoring the helix, Ro binding occurred (stem mt1 + 2, Figures 2E and 2F). Thus, while the low abundance of the Ro-bound U2 snRNA precluded structure mapping, the finding that mutations that disrupt the novel helix eliminate Ro binding, while compensatory mutations restore binding, suggests that formation of this helix is required for Ro recognition.

As the changes in the variant U2 snRNAs do not disrupt normal U2 structure, only a small fraction of the variants may misfold into the helix recognized by Ro. We therefore tested if the variant RNAs functioned in pre-mRNA splicing. We depleted endogenous U2 RNA in oocytes with oligonucleotide-directed RNase H digestion and determined whether injection of wild-type or variant U2 genes could restore splicing. Splicing was assayed by injecting a labeled splicing substrate [15]. As noted [15], lariat formation was efficient in uninjected oocytes but little spliced mRNA accumulated (Figure 2G). Depletion of endogenous U2 reduced both lariat and spliced mRNAs (Figure 2G). However, injection of genes encoding either the wild-type, variant 1, or variant 2 U2 RNAs restored splicing, increasing the output of spliced mRNAs beyond that of endogenous U2 snRNA (Figure 2G). Although this assay cannot reveal subtle differences in splicing efficiency or RNP assembly between wild-type and variant U2 RNAs, the finding that splicing is restored reveals that variant U2 snRNAs can be assembled into functional snRNPs. Thus, only a small fraction of the variant 2 snRNA may form the abnormal helix. This finding may explain why only very low levels of this RNA are found in our immunoprecipitates.

Ro Is Important for Cell Survival after Ultraviolet Irradiation

In *D. radiodurans*, a Ro ortholog contributes to survival after irradiation with UVC light [3]. We examined the viability of wild-type, *Ro*^{-/-}, and *PGK-Ro* cells after exposure to UVC. *Ro*^{-/-} cells were more sensitive to UVC irradiation than wild-type cells (Figure 3A). At 10 J/m², the highest dose, the difference was between 3- and 8-fold, depending on the experiment. *PGK-Ro* cells were more resistant to irradiation than *Ro*^{-/-} cells, confirming that some or all of the decreased survival of *Ro*^{-/-} cells was due to loss of Ro (Figure 3A). Similar to *D. radiodurans* [3], *Ro*^{-/-} cells were identical to wild-type cells in their sensitivity to ionizing radiation (Figure 3A).

Because *D. radiodurans* Ro is upregulated after UV irradiation [3], we examined whether the levels or sub-

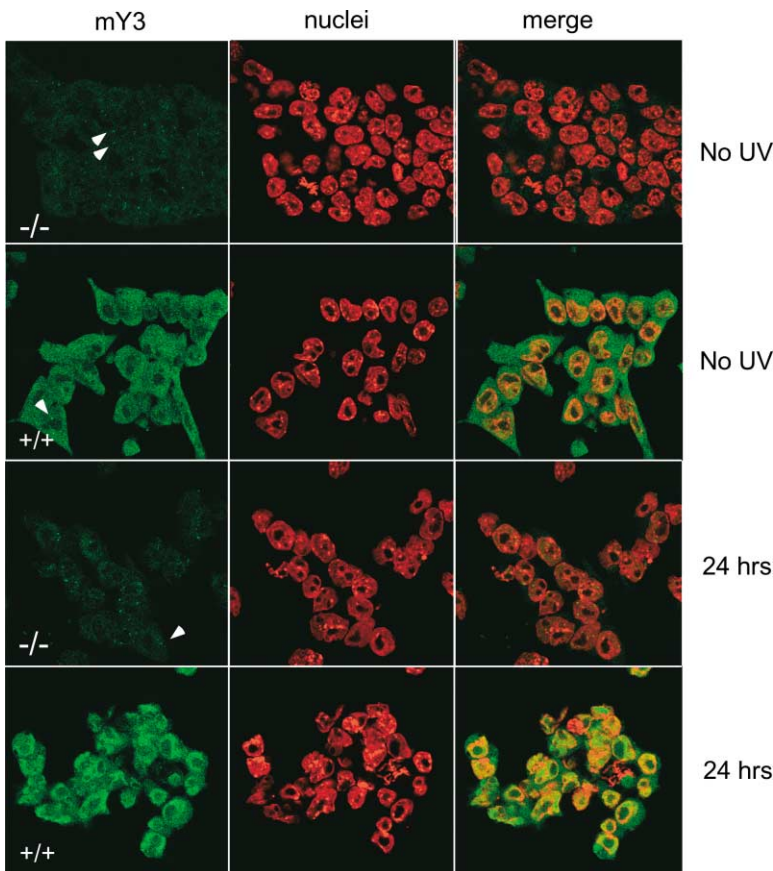


Figure 4. Y3 RNA Accumulates in Nuclei of Wild-Type Cells after UV Irradiation

Wild-type and *Ro*^{-/-} ES cells were fixed and subjected to fluorescent in situ hybridization to detect mY3. Nuclei were visualized with To-Pro-3. Cells were unirradiated (top two rows) or irradiated with 10 J/m² UVC and allowed to recover for 24 hr (bottom two rows). Arrows, punctate structures shown by double labeling to be perinucleolar compartments.

cellular distribution of mouse Ro changed. Western blotting failed to reveal differences in Ro levels (data not shown). However, immunofluorescence revealed that the Ro distribution changed. In wild-type unirradiated cells, Ro was largely cytoplasmic, although some Ro was detected in nuclei (Figure 3B). Within 2 to 3 hr of irradiation with 5 or 10 J/m² UV light, significant amounts of Ro were detected in nuclei (Figure 3B and data not shown). The intensity of nuclear staining increased with time, peaking at ~20–24 hr (Figure 3B). To determine if the change in distribution was a general phenomenon, we examined mouse embryonic fibroblasts. Ro was detected in both nuclei and cytoplasm in unirradiated cells (Figure 3C). After UV irradiation, Ro accumulated in nuclei (Figure 3C). In contrast, a control protein, the translation factor EF-2, remained cytoplasmic (data not shown).

We examined whether Y RNAs changed distribution upon irradiation. In situ hybridization revealed that mY3 RNA was found in both nuclei and cytoplasm of unirradiated ES cells (Figure 4). In both wild-type and *Ro*^{-/-} cells, we also detected small foci that were often adjacent to nucleoli (arrows). Double labeling with antibodies to hnRNP I revealed that many foci correspond to perinucleolar compartments (data not shown), subnuclear bodies that contain Y RNAs [16]. After irradiation, nuclear mY3 staining became much more prominent in wild-type cells (Figure 4; 24 hr). Thus, upon irradiation, both Ro and mY3 RNA accumulate in nuclei.

Conclusions

Our findings suggest that Ro has two conserved functions. First, Ro is likely involved in small RNA quality control, as it recognizes variant small RNAs that may be misfolded, both U2 snRNAs in ES cells and pre-5S rRNAs in *Xenopus oocytes* [2]. As the mouse genome contains many variant U2 snRNA genes [10], while *Xenopus* contains many variant 5S rRNA genes [17], the particular RNA detected bound to Ro may reflect the abundance of the misfolded RNA in the species and cell type under study. How might Ro recognize misfolded RNAs? For Y RNAs, the Ro binding site is a bulged helix. As the sequence of the helix and the presence of bulges are critical for Ro recognition, the protein likely binds in the helix major groove [18]. For misfolded pre-5S, a bulged helix is bound by Ro, but neither the sequence of the helix nor the bulge is important [4]. Thus, Ro recognition of misfolded RNAs may be distinct from Y RNA recognition. Also, while misfolded pre-5S competes with Y3 RNA for Ro binding, the relative binding affinity is 300-fold weaker [18]. Thus, one possibility is that Ro has a low affinity for RNA helices. If most helices in cells are bound by the appropriate RNA binding proteins, misfolded RNAs could be recognized by the presence of protein-free helices.

The extremely low levels of variant U2 snRNAs bound by Ro have made it difficult to determine their fate. We do not know if these RNAs eventually assemble into snRNPs or are degraded, as seen for variant pre-5S

rRNAs [2]. Moreover, nearly 1/3 of U2 snRNAs in our ES immunoprecipitates were wild-type (Table S1), raising the possibility that wild-type U2 also misfolds at low frequency. Consistent with this hypothesis, we sometimes detected low levels of wild-type U2 snRNA in our oocyte anti-Ro immunoprecipitates, particularly when increased U2 DNA was injected (X.C. and S.L.W., unpublished data). If the misfolded RNAs are folding or RNP assembly intermediates, Ro binding could assist U2 folding or the interconversion between misfolded and folded forms.

The second conserved role is that Ro promotes cell survival after UV irradiation. As shown for *D. radiodurans* lacking Ro [3], mammalian *Ro*^{-/-} cells are sensitive to UV irradiation. Irradiation causes Ro RNPs to increase in level in bacteria [3] or to accumulate in nuclei in mammalian cells, presumably by activating as yet undefined signaling mechanisms. These observations suggest a role for Ro RNPs in the recognition or repair of nuclear damage.

What is the connection between the likely role of Ro in quality control and the finding that Ro enhances survival after irradiation? In addition to inducing DNA crosslinks, UV light causes RNA:RNA and RNA-protein crosslinks [19]. Newly synthesized RNAs may be especially vulnerable. Thus, after irradiation, Ro may sequester nascent RNAs that misfold or fail to assemble into RNPs. Consistent with this idea, Ro binds variant RNAs in the nucleus, the site of Ro RNP accumulation after irradiation. However, to date we have not detected additional RNAs associated with Ro after irradiation, perhaps because damaged RNAs associate transiently or are not sufficiently abundant to be detected by our methods. Alternatively, Ro may be bifunctional, with a distinct activity important for cells to survive irradiation, such as in recovery of transcription or DNA repair. Although future experiments will be required to distinguish these possibilities and to examine the role of Y RNAs, our studies have revealed that Ro is involved in two generally important cellular processes.

Supplemental Data

Supplemental Data, including the Experimental Procedures and Table S1, are available at <http://www.current-biology.com/cgi/content/full/13/24/2206/DC1>.

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