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Regulation of c-Myc mRNA by L11 in Response to UV and Gamma Irradiation

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14. ABSTRACT In previous funding year, we have discovered a novel regulatory paradigm wherein L11 plays a critical role in controlling <i>c-myc</i> mRNA turnover via recruiting miR-24-loaded miRISC to the <i>c-myc</i> mRNA 3'-UTR in response to ribosomal stress. We show that ribosome-free L11 binds to <i>c-myc</i> mRNA in the cytoplasm and this binding is enhanced in response to ribosomal stress. Meanwhile, we found that <i>c-myc</i> mRNA is also down-regulated in response to DNA damage including UV and γ -irradiation in an L11-dependent manner. In current funding year, we have explored the role of miR-130a in DNA damage-mediated <i>c-myc</i> down-regulation. We provided evidence indicating that miR-130a directly targets <i>c-myc</i> mRNA. UV treatment enhances the association of L11, Ago2, as well as miR-130a with the <i>c-myc</i> mRNA. Together, our current results suggest that L11 may recruit miR-130a-loaded miRISC to mediate <i>c-myc</i> decay in response to UV-induced DNA damage and implying that miR-130a may possess a growth-inhibitory function through down regulating c-Myc.					
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A. INTRODUCTION

The c-Myc oncoprotein is deregulated in many human cancers. Thus, proper control of c-Myc level and activity is essential for normal cell growth and proliferation. We have previously identified that ribosomal protein L11 suppresses c-Myc transactivation activity^(1,2) and reduces *c-myc* mRNA levels⁽³⁾. Interestingly, *c-myc* mRNA is markedly reduced by treatment of ribosomal stress-inducing agents actinomycin D and 5-fluorouracil. During this funding period, we have found that L11 plays a novel and key role in mediating ribosomal stress-induced *c-myc* mRNA turnover⁽⁴⁾. Interestingly, c-Myc is also down-regulated in cells following DNA damage, such as those induced by ultraviolet (UV) and γ -irradiation (IR). The purpose of this proposal is to examine whether and how L11 is involved in the regulation of c-Myc in response to DNA damage. Specifically, we will determine whether L11 regulates *c-myc* mRNA levels and stability by recruiting miRNAs in response to UV and γ -IR as well as the mechanism underlying the L11 regulation of *c-myc* mRNA. Results from these experiments would demonstrate an important function of L11 in regulating *c-myc* mRNA in response to DNA damage and offer useful information for developing anti-tumor drugs that target *c-myc* mRNA in cancers and thus have a significant impact on the understanding of c-Myc-induced tumorigenesis.

B. BODY

During the past two funding years, we have characterized a novel mechanism of L11 regulation of *c-myc* mRNA stability. We have found that L11 recruits microRNA (miRNA)-24 (miR-24) loaded RNA interference silencing complex (miRISC) to suppress *c-myc* mRNA expression. L11 binds to the *c-myc* mRNA at its 3'-untranslated region (3'-UTR). Overexpression of L11 suppresses the expression of luciferase mRNA and activity, whereas knockdown of L11 increases these levels and activity, in cells transfected with luciferase reporter containing the *c-myc* 3'-UTR (pGL3-myc 3'UTR), but not the control pGL3 vector. We further confirmed that L11 binds to the miRISC component Ago2 and miR-24. Knockdown of L11 rescued the *c-myc* mRNA reduction mediated by either overexpression of miR-24 or knockdown of Ago2, suggesting that L11 recruits miR24/miRISC to repress c-Myc. Interestingly, ribosomal stress induced by perturbation of ribosomal biogenesis results in a significant *c-myc* mRNA reduction in a L11-dependent manner in cells. L11 binding to *c-myc* mRNA, miR-24, and Ago2 was significantly increased following ribosomal stress. We further demonstrated that it is the ribosome-free L11 that binds to the *c-myc* mRNA in the cytoplasm. Ribosomal stress triggers the release of L11 from the nucleolus to both the nucleoplasm, where it binds to c-Myc protein, and the cytoplasm, where it binds to *c-myc* mRNA. Importantly, we show that L11 regulation of *c-myc* mRNA is specific as several other tested ribosomal proteins do not regulate *c-myc* mRNA levels. Altogether, our data identify a novel regulatory paradigm wherein L11 plays a critical role in controlling *c-myc* mRNA turnover via recruiting miRISC in response to ribosomal stress, thus ensuring a tight coordination between the levels and activity of c-Myc and ribosomal biogenesis. This work has been published in *Mol Cell Biol* (2011)⁽⁴⁾.

In addition, we have purified L11-associated-miRNAs and mRNAs from 293 cells using deep sequencing. Our initial results identified that L11 associates with a number of novel miRNAs (see below), including miR-130a in addition to miR-24, and mRNAs (including L11 itself, ctBP, Bcl-2, etc.) Now we have evidence indicating that miR-130a may regulate c-Myc by directly targeting *c-myc* mRNA (See below).

Specifically relating to the statement of Work (SOW) of this award, following points are either addressed or under planning:

Aim 1. To determine if L11 regulates c-myc mRNA in response to UV and γ -IR.

This aim was **completed** during the first funding year (please see progress report for the previous year). We have also expanded the experiments by focusing on UV irradiation and confirmed that UV treatment decreases the levels of c-Myc protein and *c-myc* mRNA in a dose-dependent manner (data not shown). This reduction requires L11 as it was abolished by L11 knockdown in cells. Thus L11 plays an important role in regulating *c-myc* levels following DNA damage (please see last years' progress report).

Aim 2. To examine if L11 recruits miRNA(s) to the 3'UTR of c-myc mRNA in response to UV and γ -IR.

During the first funding year, we have found that UV damage enhances L11 association with *c-myc* mRNA (**task 2(1)**). We further showed that L11 binding to the 3'-UTR of *c-myc* mRNA was induced by UV treatment, suggesting that L11 regulates *c-myc* mRNA levels by acting on *c-myc* 3'-UTR in response to UV-induced DNA damage (please see last years' progress report for the previous years).

During this funding year, we have focused on the role of miR-130a in regulating c-Myc levels following DNA damage. Our RNA-IP-RNAseq assays from 293 cells stably expressed Flag-L11 using anti-Flag antibody (part of **task 2(5)**) showed that miR-130a is one of the L11-associated miRNAs (**Fig. 1**). Bioinformatics prediction using TargetScan (<http://www.targetscan.org>) identified three putative seedless miR-130a-binding elements at the *c-myc* 3'-UTR (**Fig. 2**). We further confirmed the association of L11 with miR-130a in 293 and U2OS cells (**Fig. 3**), suggesting that L11 may recruit miR-130a to target *c-myc* mRNA.

To further characterize the role of miR-130a in c-Myc regulation, we have performed experiments covered in part in **tasks 2(2)-2(4)**. We focus on miR-130a instead of the proposed miR-145 or let-7, as miR-130a is potentially a tumor suppressive miRNA and therefore the finding of c-Myc targeting by miR-130a would be extremely novel. We first tested whether miR-130a targets *c-myc* mRNA. As shown in **Fig. 4**, overexpression of miR-130a mimics significantly reduced the levels of c-Myc protein and *c-myc* mRNA. To further test whether miR-130a targets *c-myc* 3'-UTR, we performed luciferase reporter assays. As shown in **Fig. 5**, overexpression of miR-130a significantly reduced the luciferase activity in cells transfected with pGL3-myc 3'UTR, but not the control pGL3, reporter plasmid. Also, Overexpression of miR-130a significantly increased the binding of Ago2 to the *c-myc*, but not *GAPDH*, mRNA (**Fig. 6**). Altogether, these data strongly indicate that miR-130a might directly target the *c-myc* mRNA to regulate the levels of c-Myc.

miR-16	miR-103
miR-1248	miR-130a (+/-)
miR-3944 (-)	miR-1285(+/-)
miR-191	miR-1181 (-)
miR-548d-3p (-)	miR-320a
miR-1972 (+/-)	hsa-miR-24
miR-1244	miR-107
miR-744	miR-1234 (-)

Figure 1. L11-associated miRNAs identified by RNA-IP-RNAseq. 293 cells transfected with Flag-L11 were subjected to affinity purification of L11-RNA complex followed by RNAseq.

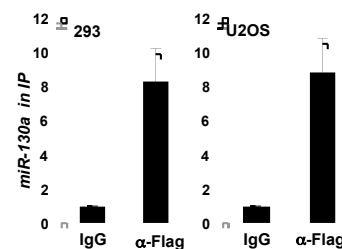


Figure 3. L11 binds to miR-130a. 293 and U2OS cells were transfected with Flag-L11 and subjected to IP using anti-Flag antibody or control IgG followed by RT-qPCR detection of *miR-130a*.

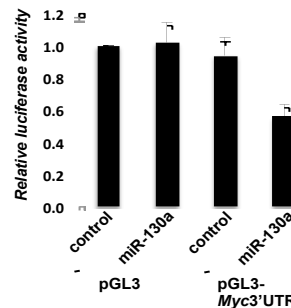


Figure 5. Overexpression of miR-130a reduces the luciferase activity controlled by the *c-myc* 3'-UTR. U2OS cells transfected with β-gal and pGL3 or pGL3-myc3'UTR plasmids together with control or miR-130a mimics were assayed for the relative luciferase activity normalized to β-gal expression.

---UCCUUCUACAGAAAUGUCCUG))))))))) UACGGGAAAUAUGUA---ACGUGAC	-26.1 Kcal/mol (nt 21-42)
-UGCAACCUCACAACCUUG-GCUG)))))))))) UACG---GGAAAUAUGUAACGUGAC	-23.1 Kcal/mol (nt 82-103)
AUGAACUUGUUUCAAUGCA-UG)))))))))) UACGGGAAAUAUGUA-ACGUGAC	-20.5 Kcal/mol (nt 54-75)

Figure 2. Prediction of the miR-130a binding sites at *c-myc* 3'-UTR. TargetScan prediction of the miR-130a targeting sequences is shown in the left column. The calculated folding energy and the positions of targeting sequence are shown on the right column.

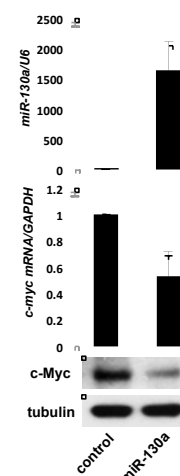


Figure 4. Overexpression of miR-130a decreases the c-Myc levels. U2OS cells transfected with control or different doses of miR-130a mimics were assayed for the relative expression of miR-130a normalized with U6 snRNA (top panel), *c-myc* mRNA normalized with *GAPDH* mRNA (middle panel) using RT-qPCR assays, as well as c-Myc protein (bottom panel) using IB.

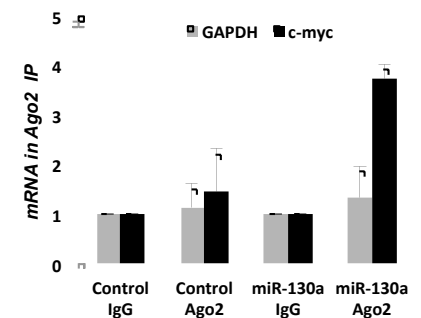


Figure 6. Overexpression of miR-130a enhances the recruitment of Ago2 to the *c-myc* mRNA. U2OS cells transfected with control or miR-130a mimics were subjected to RNA IP with anti-Ago2 antibodies, followed by RT-qPCR assays to determine the levels of *GAPDH* and *c-myc* mRNA in the immunoprecipitates.

Aim3. To elucidate the mechanism underlying L11 regulation of *c-myc* mRNA in response to UV and γ-IR.

To further examine whether miR-130a targets *c-myc* following UV treatment, we performed RNA-IP assays. Our initial results indicate that UV treatment significantly increased the binding of Ago2 to both *c-myc* mRNA (**Fig. 7**) and miR-130a (not shown) in cells (as proposed in **task 3(1)**). Interestingly, UV treatment induced the levels of miR-130a in cells (**Fig. 8**), suggesting that the increased recruitment of miR-130a-loaded

miRISC to *c-myc* mRNA might be due to increased levels of miR-130a expression. It is interesting to test whether L11 is required for this increase of

miR-130a expression following UVC treatment, and if yes, whether L11 plays a role in the miR-130a biogenesis--- the processing of pri- or pri-miR-130a to mature miR-130a. Supporting the possible role of L11 in miRNA biogenesis, we have found that L11 interacts with DGCR8 (Fig. 9) and Drosha (Fig. 10), two core components of the microRNA microprocessor complex.

We are currently investigating the role of L11 in regulating miR-130a biogenesis in response to UV-induced DNA damage as well as its implication in miR-130a-mediated down-regulation of *c-myc* upon UV treatment. We will also explore whether other RNA-binding proteins could participate in the L11 regulation of the *c-myc* mRNA stability in response to UV treatment as proposed in aim3. We expect that at the end of the funding period, a manuscript summarizing above finding will be ready for publication.

C. KEY RESEARCH ACCOMPLISHMENTS:

- (1). L11 destabilizes *c-myc* mRNA via a miRNA-mediated pathway.
- (2). *c-myc* mRNA is reduced in response to DNA damage (UV or IR) and ribosomal stress.
- (3). DNA damage or ribosomal stress-induced *c-myc* mRNA down regulation requires L11.
- (4). miR-130a targets *c-myc* mRNA in cells.
- (5). UV treatment increased the levels of miR-130a expression
- (6). L11 interacts with the miRNA microprocessor complex Drosha-DGCR8.

D. Reportable Outcomes.

- (1) Manuscript: This award supports the following manuscripts:

(i). Challagundla KB, Sun XX, Zhang X, DeVine T, Zhang Q, Sears R, **Dai MS**. (2011) Ribosomal protein L11 recruits miR-24/miRSIC to repress c-Myc in response to ribosomal stress. *Mol Cell Biol*, 31(19): 4007-4021

(ii). Sun X-X, DeVine T, Challagundla KB, **Dai M-S**. (2011) Interplay between ribosomal protein S27a and MDM2 in p53 activation in response to ribosomal stress. *J Biol Chem*, 286(26): 22730-22741

(iii). Sun X-X, Challagundla KB, **Dai M-S**. (2012) Positive regulation of p53 stability and activity by the deubiquitinating enzyme Otubain 1. *EMBO J*, 31(3): 576-592

- (2) Employment/training. This award supports one postdoctoral in the lab for his employment and training.

E. CONCLUSIONS

L11 plays an important role in *c-myc* downregulation in response to DNA damage, suggesting that microRNA-mediated *c-myc* mRNA decay is an important mechanism that coordinates ribosomal biogenesis and c-Myc activity during stress conditions.

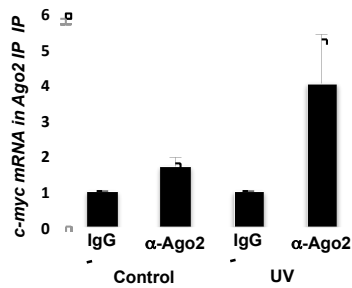


Figure 7. UV treatment enhances the recruitment of Ago2 to the *c-myc* mRNA. U2OS cells treated with control or UVC (40J/m²) for 5 hours were subjected to RNA IP with anti-Ago2 antibodies, followed by RT-qPCR assays to determine the levels of *c-myc* mRNA.

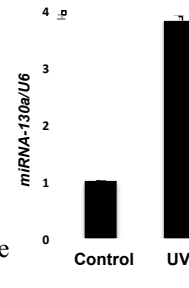


Figure 8. UV treatment increases the levels of miR-130a. U2OS cells treated without or with UVC (40J/m²) for 5 hours were assayed by RT-qPCR detection of miR-130a.

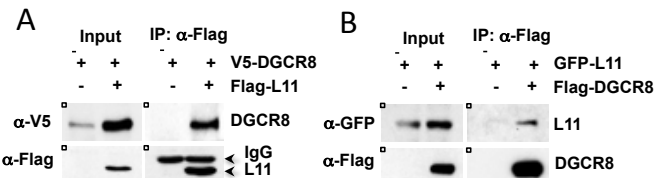


Figure 9. L11 interacts with DGCR8. 293 cells transfected with V5-DGCR8 and Flag-L11 or control vector (A) or with GFP-L11 and Flag-DGCR8 or control vector (B) were subjected to co-IP with anti-Flag followed by IB.

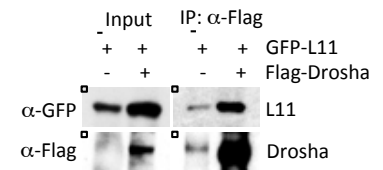


Figure 10. L11 interacts with Drosha. 293 cells transfected with GFP-L11 and Flag-Drosha or control vector were subjected to co-IP with anti-Flag followed by IB.

F. REFERENCE.

1. **Dai MS**, Arnold H, Sun XX, Sears R, Lu H. (2007) Inhibition of c-Myc activity by ribosomal protein L11. *EMBO J*, 26: 3332-3345
2. **Dai MS**, Sun XX, Lu H. (2010) Ribosomal protein L11 associates with c-Myc at the 5S rRNA and tRNA genes and regulates their expression. *J Biol Chem*, 285(17): 12578-12594
3. **Dai MS**, Sears R, Lu H. (2007) Feedback inhibition of c-Myc by ribosomal protein L11. *Cell Cycle*, 6: 2735-2741
4. Challagundla KB, Sun XX, Zhang X, DeVine T, Zhang Q, Sears R, **Dai MS**. (2011) Ribosomal protein L11 recruits miR-24/miRSIC to repress c-Myc in response to ribosomal stress. *Mol Cell Biol*, 31(19): 4007-4021

G. APPENDICES

The article by Challagundla KB et al. *Mol Cell Biol*, 31(19): 4007-4021 (2011) was attached in last years' annual progress report.

H. SUPPORTING DATA

N/A