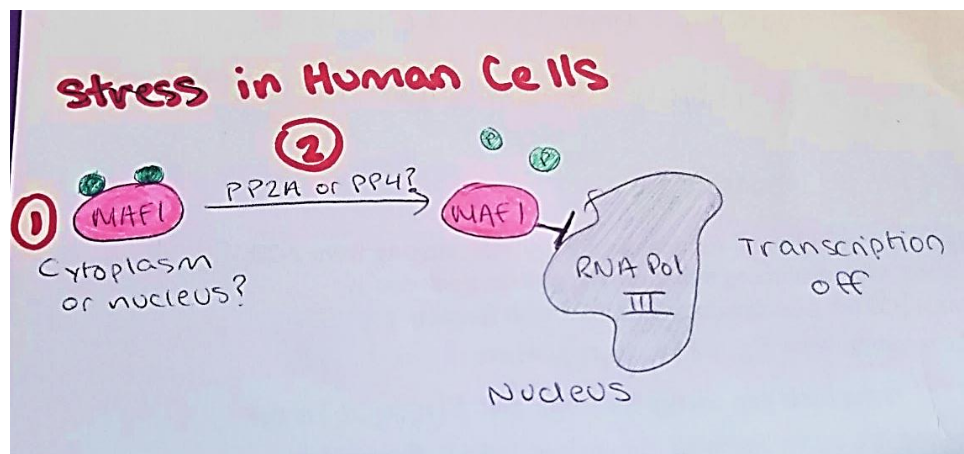
85/100, Your writing is consistently strong and clear, you found an important experimental question, and your experiments are on the mark. My comments on phrasing are not criticisms, but compliments in disguise intended to make your descriptions even clearer. My main concern is not the techniques, but not clearly saying how many MAF1 phosphorylation sites there are — if it's known); how many kinases are potential culprits — each could have different site specificity; and whether phosphorylation is all or nothing, or could be partial or site-specific under different conditions. You might not be able to address all these questions, but it's part of expanding your reader's understanding of this deceptively simple problem.



RNA polymerase III transcribes transfer RNAs as well as other essential non-translated RNAs including snRNA, miRNA, and 5s rRNA. Pol III dysregulation is common in cancer and has been associated with tumorigenesis. As a result, understanding Pol III regulation could be key to elucidating potential therapeutic targets. One important regulator is MAF1, a Pol III repressor in stress conditions such as starvation or DNA damage. Phosphorylated MAF1 is inactive, while dephosphorylated MAF1 inhibits Pol III activity. Though the mechanism of yeast MAF1 activation has been studied, human MAF1 is not well understood. In the following experiments, I propose to understand which phosphatase is responsible for regulating human MAF1, and whether dephosphorylated MAF1 localizes in a different part of the cell than phosphorylated MAF1. Understanding the regulation and localization of human MAF1 ultimately may be important in helping find therapeutic targets for cancer treatment.

nice, totally clear and to the point

Introduction

In eukaryotes, the transcription of genomic DNA is carried out using three different multi-subunit complexes: RNA Polymerases I, II, and III. RNA polymerase III is a highly evolutionarily conserved complex consisting of 17 subunits [1]. The Pol III enzyme transcribes

transfer RNAs (tRNAs) and many other essential components of protein biosynthetic machinery, including 5s rRNA, small nuclear RNA (snRNA), micro RNAs (miRNA), RNase P RNA, and 5s rRNA [2, 3]. Pol III transcription rates are tightly regulated in response to different cellular conditions. Defects and dysregulation of Pol III-dependent transcription are common phenomena in cancer. Specifically, Pol III hyperactivity has been associated with tumorigenesis in mammals [7]. Thus, understanding the regulation of Pol III function is important both for normal physiology, and cancer.

For unicellular eukaryotes such as yeast (*Saccharomyces cerevisiae*), the coordination of tRNA levels and ribosome function is vital for optimal nutrient utilization and survival. Pol III-dependent tRNA transcription is down-regulated under conditions of nutrient starvation [4], secretion defects, DNA damage, and chemical treatment [5, 6]. These signals that repress Pol III converge on a central negative regulator: the MAF1 protein [8]. Mechanistically, when MAF1 is active it inhibits TFIIIB assembly, thereby inhibiting Pol III-dependent transcription (Fig. 1) [10]. This is also supported by the three-dimensional crystal structure of MAF1and cryo-electron microscopic structure of Pol III, which show that MAF1 binds to the Pol III clamp and blocks recruitment of Pol III to the complex of BRF1-TBP-promoter DNA [12].

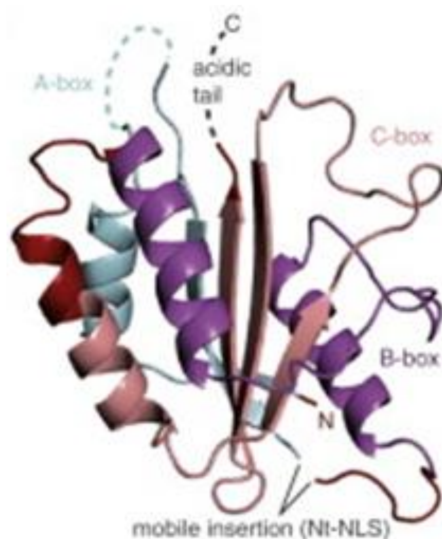


Fig. 1. MAF1 ribbon model. Conserved boxes A, B, and C highlighted in blue, purple, and rose respectively [12].

should say there are multiple phosphorylation sites if that is the case... this would prepare your reader for multiple kinases in the following sentences, and possibly for multiple phosphatases if that turned out to be the case.

Though MAF1's role as a repressor of Pol III activity is conserved in a variety of eukaryotic organisms [13, 14], its regulation still requires further study. MAF1 is regulated through post-translational modifications. It is hyper-phosphorylated during normal cell growth and becomes hypo-phosphorylated under stress conditions [16-18]. In yeast, several different kinases regulate MAF1 phosphorylation, including rapamycin complex 1 (TORC1) and casein kinase 2 (CK2) [15]. MAF1 is also phosphorylated by protein kinase A and Sch9, a ribosomal S6 kinase (S6K) homolog. In yeast, MAF1 is found predominantly in the cytoplasm under optimal growth conditions. During stress, MAF1 is rapidly dephosphorylated and accumulates in the nucleus [16]. This is significant, because without nuclear accumulation MAF1 is unable to interact with TFIIIB and repress Pol III transcription. MAF1 phosphorylation also appears to affect its binding affinity for Pol III [16-18]. The yeast MAF1 phosphatase, identified in the past as protein phosphatase 2 (PP2A), is shown as protein phosphatase 4 (PP4) in a recent study [20].

While yeast MAF1 has been studied extensively, comparatively little is known about human MAF1. In humans, mTORC1 is the only kinase that has been shown to phosphorylate MAF1 definitively [21]. Like yeast, human MAF1 treated with mTORC1 inhibitor rapamycin becomes largely dephosphorylated and associates with Pol III [19]. However, the localization of human MAF1 within the cell, depending on its phosphorylation, has not been well studied. Whether human MAF1 contains a nuclear localization signal is also unclear. Further, it is unknown which phosphatase(s) specifically dephosphorylates MAF1. The aims of this study include determining whether human MAF1 undergoes cytoplasmic-nuclear shuttling in a comparable way to yeast MAF1. This study further aims to elucidate whether human PP2A or PP4 primarily dephosphorylate MAF1, and whether that dephosphorylation affects MAF1

This is *the* pivotal sentence before your aims, but it sounds strange because "is shown" is so vague and mysterious... it could mean that there is more than 1 phosphatase, or that PP2A is called PP4 in a different organism, or that the studies clash.

"the" seems odd because it implies that there is only 1 phosphatase yet so many kinases, unless there is only 1 phosphorylation site, or "hypo-/hyper-phosphorylated" refers to fractional phosphorylation rather than differential phosphorylation of multiple sites.

localization. A better understanding of human MAF1 may be important in finding therapeutic targets for cancer treatment.

Experimental Methods

1. Cell culture and transfection

To analyze MAF1 phosphorylation sites and localization, we will generate a HeLa spinner cell line (referred to as the DBR28 cell line) expressing MAF1 tagged with Flag and His tags at its C terminus. This cell line has been used successfully in past experiments studying human MAF1. We will grow two samples of DBR28 cells as well as two samples of control spinner HeLa cells that do not express tagged MAF1.

not totally clear whether this means the 2 phosphatases could not be distinguished experimentally, or that both dephosphorylate hMAF1

2. Co-Immunoprecipitation

Since MAF1 dephosphorylation is a common mechanism in all reports of rapid MAF1-dependent repression of Pol III to date, the precise identity of the phosphatase(s) and how it/they are regulated is the central question of this study. In yeast cells, past work has suggested MAF1 interaction with either PP2A or PP4 [20]. To determine the interaction of MAF1 with particular phosphatases in human cells, both in stress and nonstress conditions, we aim to utilize co-immunoprecipitation (co-IP). Treatment with the DNA-damaging agent MMS for 30 minutes will serve as a stress treatment, activating the relevant phosphatase. Using appropriate polyclonal antibodies, we will then co-precipitate MAF1 with PP4 or MAF1 with PP2A in both unstressed and MMS treated cells. Cells transformed with tagged MAF1 and/or PP4 or PP2A will be isolated after a 15-minute period of formaldehyde crosslinking. Extracts will then be incubated with FLAG beads to immunoprecipitate either PP4 bound to MAF1 or PP2A bound to MAF1.

not totally clear whether these are against MA1 or PP2 OR PP4

I don't understand... are you expecting MAF1 to co-IP with the physiological phosphatase? The phosphatase must bind to phosphorylated MAF1, but unless dephosphorylation is slow or somehow blocked, the 2 proteins will probably dissociate after dephosphorylation. Instead, could you treat cells with phosphatase inhibitors like okadaic acid or calyculin before opening them for co-IP [see for example Nifoussi et al. (2014) J Biol Chem 289, 21950?

this does not clarify what the antibodies are against... Are the tags for the co-IP or for the Western blot that follows?

does "level" mean fractional phoshorylation or particular site of phosphorylation?

We will then determine if MAF1 is enriched in either of the phosphatase containing extracts and compare them to extracts containing only MAF1.

3. Immunofluorescence

Yeast MAF1 has shown cytoplasmic-nuclear shuffling depending on its phosphorylation level. To determine if human MAF1 is similarly regulated at the level of its intracellular localization, we will use a fluorescent FLAG antibody to localize MAF1 in HeLa cells expressing tagged MAF1 by immunofluorescence. The antibodies' specificity will be determined by imaging control HeLa cells that have not been transformed and these cells should not show any labeling. We will also use DAPI nuclear staining, and then merge with images of MAF1-flag fluorescence. Overlap will imply MAF1 nuclear localization. The percentage of cells with nuclear MAF1 signal clearly above the cytoplasmic signal will be measured.

We will conduct this imaging both in the presence and absence of rapamycin, an mTOR inhibitor, which should effectively prevent the phosphorylation of human MAF1. If human MAF1 is shown to localize in a similar manner as yeast MAF1 we would expect to see MAF1 signal in the cytoplasm in the absence of rapamycin, and in the nucleus in the presence of rapamycin. If this hypothesis is supported, we will then use the fluorescent FLAG antibody to localize MAF1 in the presence of shRNA for PP4 and PP2A respectively to determine whether the lack of function of either phosphatase impacts MAF1 localization.

nice... this in vivo experiment will nicely complement your in vitro IPs

4. Northern Blots to compare Pol III transcription

To compare the effect of either PP4 or PP2A inactivation not only on MAF1 but on Pol III transcription, we will perform a Northern blot. We will infect HeLa cells expressing tagged MAF1 with shRNA for a control vector, PP4, or PP2A. We will first perform a Northern blot to confirm reduced repression of either PP4 or PP2A We will then perform a Northern blot to

Is there a published precedent for doing Northerns on this particular tRNA precursor? One might worry that the precursor would be scarce, or be processed so quickly that you could not see it.

investigate the change in pre-$tRNA^{Leu3}$ transcription in the presence and absence of MMS. We expect to see pre-$tRNA^{Leu3}$ attenuation in the control lane. If PP4 or PP2A interact with MAF1, we would not see attenuation in the lanes for their respective shRNAs in the presence of MMS.

5. *In vitro* dephosphorylation assay

citing a published precedent is the best way to dispel doubts

While co-immunoprecipitation and immunofluorescence are useful tools for determining MAF1 interaction with phosphatases, they do not clearly determine whether PP4 or PP2A directly dephosphorylate MAF1. To do so, we will conduct an *in vitro* dephosphorylation assay. We aim to isolate and purify phosphorylated MAF1 from HeLa cells transfected with tagged MAF1 using the QIAGEN phosphoprotein purification protocol. We will then compare dephosphorylation of phosphorylated MAF1 when incubated with PP4 or PP2A for 2 or 8 hours, in the presence or absence of MMS. Dephosphorylation can be visualized on a Western Blot, with dephosphorylated species running farther down the gel. We will use λ phosphatase as a positive control for a highly active (though nonspecific) phosphatase enzyme. To confirm and calibrate PP4 and PP2A activity, we will isolate phosphorylated Rad53, a known native substrate of PP4 [22], and Net1 a known native substrate of PP2A [23]. We expect to quantify the change in Rad53 and Net1 mobility to calculate λ phosphatase activity as a control (by comparing the change in the band as a percentage of total Rad53 signal per lane).

are there precedents for preservation of phosphatase specificity in vitro, or could a low level of nonspecific activity mislead if you didn't do a thorough time course with the right controls?

6. EdU Proliferation Assay

To further investigate how dephosphorylation of MAF1 by either PP4 or PP2A affects Pol III transcription, we will also conduct a cell proliferation assay. We hypothesize lower cell proliferation when cells with active, dephosphorylated MAF1 are present in stress conditions. We plan to infect HeLa cells with control shRNA, shRNA for PP4, shRNA for PP2A, as mentioned above and we will perform a Northern Blot to confirm shRNA function. We will then

might be good to distinguish between proliferation and survival... the phenotype of misregulated MAF1 may be greater stress and apoptosis

measure cell proliferation over time using an EdU Assay (Fig. 2), in the presence and absence of MMS. This proliferation assay may be particularly useful in identifying a particular MAF1 phosphatase as a therapeutic target to prevent tumorigenesis.

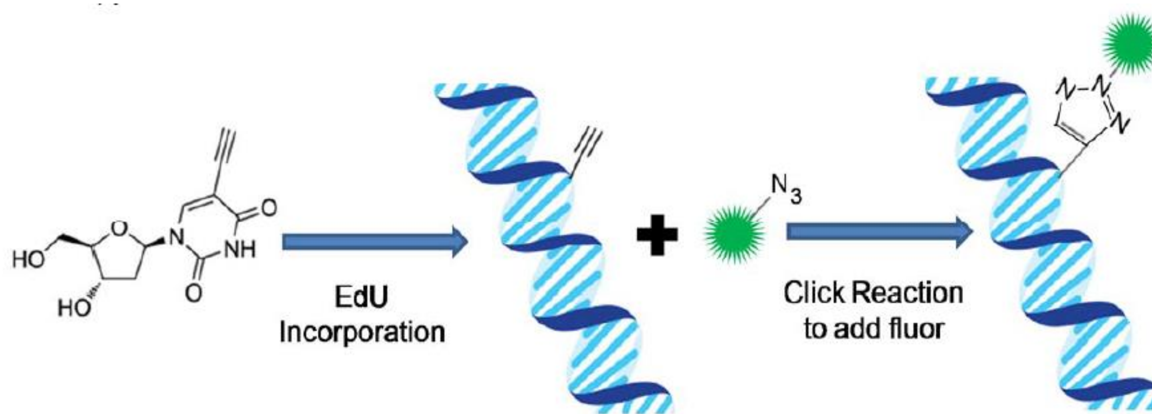


Fig. 2. EdU Cell Proliferation Assay Principle. Cells grown in the presence of 5-EdU incorporate the compound at thymidine bases during S-phase. Fluorophore-labeled azide reacts with the incorporated EdU to allow detection by microscopy or flow cytometry. (EdU Cell Proliferation Assay Instructions for Use, Millipore Sigma).

I wasn't familiar with this assay, and it's not obvious how it assays proliferation... a few words of explanation can spare your readers a lookup when they're steaming along

Conclusion

RNA polymerase III is important to cellular function as it transcribes transfer RNAs as well as other essential non-translated RNAs including snRNA, miRNA, and 5s rRNA. Pol III regulation is complex, but one key regulator appears to be Pol III repressor MAF1, which is activated under stress conditions. The study of yeast MAF1 has shown it to be itself complex, localizing in different cellular compartments under different conditions, and interacting with the mTOR kinase. However human MAF1 is still not well understood. We have proposed the above experiments to understand which phosphatase(s) interacts with human MAF1, and whether human MAF1 is shuttled from the cytoplasm to the nucleus under stress conditions. Ultimately, understanding the regulation of MAF1 and its interaction with Pol III in human cells will be

important to mitigating dysregulation is associated with tumorigenesis, and uncovering potential therapeutic targets in this pathway.

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