During transcription, a variety of stressors can induce RNA Polymerase II (RNAPII) arrest. Normally this stalling is rescued, allowing transcription to continue. However, if polymerase rescue fails transcription can instead be terminated by the degradation of Rpb1, the largest subunit of RNAPII\(^1\). While the mechanism determining how cells regulate entrance into this last resort polymerase degradation pathway is unknown, the decision involves activation of the protein Def1. Def1 binds Rpb1 and recruits the ubiquitin ligase complex that polyubiquitinates Rpb1, targeting it for degradation via the proteasome. In response to UV damage, the C-terminus of Def1 is proteolytically cleaved, resulting in Def1 accumulation in the nucleus and induction of the ubiquitination of Rpb1 (Fig 1). The C-terminus of Def1 appears to promote cytoplasmic localization and cleavage of Def1 is sufficient for its activation\(^2\). How Def1 activation is regulated in response to transcriptional stress remains an open question.

The ubiquitin ligase Rsp5 monoubiquitinates Rpb1 in response to transcriptional stress and likely intrinsically recognizes a stalled polymerase\(^3,4,5\). Rsp5 also ubiquitinates Def1, mediating its proteolytic processing\(^2\). While the majority of Def1 is cytoplasmic in the unstressed state, a small portion of Def1 is always nuclear-localized and binds Rpb1\(^2,6\) (Fig 1). The dual role of Rsp5 in ubiquitinating Rpb1 and Def1 presents an attractive model for Def1 activation, where Rsp5 binding to Rpb1 might facilitate Def1 activation (Fig 2). In this case, recognition of polymerase arrest would promote Def1 activation at the stalled polymerase. However, Rsp5 is localized to both the nucleus and cytoplasm\(^7\). Thus, an independent model contends that transcriptional stress may stimulate Rsp5 ubiquitination of Def1 in the cytoplasm (Fig 3).

It is unknown if Def1 is generally activated in response to transcriptional stress or if activation is more tightly regulated. If transcriptional stress indiscriminately induces Def1 activation, secondary regulatory mechanisms may inhibit Def1 function until polymerase degradation becomes necessary. In line with this, Def1 forms a chromatin-associated complex with Rad26, a SWI/SNF ATPase required for transcription-coupled repair\(^8,9\). The role of this complex is unknown, but genetic evidence indicates that Rad26 antagonizes Def1 function\(^8\). Def1 and Rad26 exist in competing pathways for responding to transcriptional arrest. Thus, this complex likely plays an inhibitory role, protecting Rpb1 from degradation while other repair mechanisms are attempted\(^10\). I hypothesize that regulation of Def1 is two-fold: coupling of activation to recognition of transcriptional arrest and subsequent inhibitory protein-protein interactions confine Rpb1 degradation to last resort scenarios.

**AIM1: Investigate if recognition of arrested polymerases mediates nuclear Def1 activation**

Rsp5 preferentially ubiquitinates Rpb1 in a stalled elongation complex\(^3\). Thus, Rsp5 ubiquitination of Rpb1 may be an intrinsic mechanism for recognizing arrested polymerases during transcriptional stress\(^1\). This nuclear Rsp5 function may link recognition of stalled polymerases to Def1 activation. To test this, I will determine if binding of Rsp5 to Rpb1 is necessary for Def1 activation. Phosphorylation of Ser-5 on Rpb1 inhibits Rsp5 binding. I will employ a temperature sensitive mutant of ssu72, the phosphatase that removes Ser-5 phosphorylation, to inhibit Rsp5 binding and ubiquitination of Rpb1\(^5\). Def1 activation will be quantified via cleavage, identifiable by western blot\(^2\). If Def1 is not cleaved in the ssu72 mutant at the non-permissive temperature in response to UV, then Rsp5 binding to Rpb1 is necessary for Def1 activation. To test if Rsp5 binding to Rpb1 is also sufficient for Def1 activation, I will overexpress a nuclear localized Rsp5 to determine if this increases Def1 activation. As a control, I will ensure that this overexpression corresponds to an increase in Rpb1 monoubiquitination in both wildtype and Def1 deletion strains. I will localize Rsp5 to the nucleus by mutating both the nuclear exit signal (NES) and Crm1, a nuclear export receptor that mediates Rsp5 export, but not Def1 export\(^5,7\). This experiment would also test if depletion of cytoplasmic Rsp5 decreases Def1 activation. As Def1 is hypothesized to shuttle between the nucleus and cytoplasm, activation could occur in the nucleus or the cytoplasm\(^2\). To probe the
role of localization in Def1 activation, I will add additional NES sequences to Rsp5 to drive cytoplasmic localization and see if this alters the level of Def1 activation. Similarly, I will also append an NLS or NES to Def1 in order to drive nuclear or cytoplasmic Def1 localization. These constructs will be GFP-tagged to confirm localization. If activation occurs in the nucleus, I would expect nuclear localization of both Def1 and Rsp5 to increase Def1 cleavage levels, whereas cytoplasmic localization would decrease cleavage. I would expect the opposite results if activation occurs in the cytoplasm. However, nuclear function of Rsp5 may still be a prerequisite for cytoplasmic Def1 activation. As there may be residual expression of these constructs, these experiments can only identify if a specific Def1/Rsp5 localization enhances or decreases Def1 activation, but cannot establish localization requirements.

The above experiments test if the Rsp5-Rpb1 interaction and Rsp5/Def1 nuclear localization facilitate Def1 activation. While this would be consistent with a nuclear activation model, this would not be incongruent with cytoplasmic models. To distinguish between these possibilities, I will test if Def1-Rpb1 binding is a pre-requisite for activation. This would suggest that Def1 cleavage occurs at the stalled polymerase, which is not a requirement in cytoplasmic models. Since nuclear Def1 binds Rpb1, binding of Rsp5 to Rpb1 may bring Rsp5 in close proximity to Def1, facilitating ubiquitination. To test this, I will mutate candidate Rpb1 residues and conduct \textit{in vitro} binding assays with Def1 to identify and disrupt the binding interface of Rpb1 and Def1. I will then test if these Rpb1 mutants inhibit Def1 activation in response to UV \textit{in-vivo}. If binding is not a prerequisite for Def1 activation, then cleaved Def1 should still be observed. In this case, nuclear activation is still possible, but is likely mediated by other unidentified factors. Taken together, these experiments will clarify where Def1 activation is localized and establish if both Rsp5 and Def1 binding to RNAPII are requirements for Def1 activation. These interactions would support a model where recognition of arrested polymerase via Rsp5 may trigger Def1 activation at the arrested polymerase. If the results support a cytoplasmic model, I will utilize immunoprecipitation and mass spectrometry to explore how transcriptional stress may modify cytoplasmic Rsp5 to induce ubiquitination of Def1.

**AIM 2: Elucidate the role of the Rad26-Def1 complex in regulating Def1 activity**

After Def1 activation, inhibition of Def1 function may play an additional regulatory role by allowing other pathways a chance to rescue arrested polymerases. The Rad26-Def1 complex is a likely candidate to mediate such inhibition. ∆Rad26 mutants have increased Rpb1 degradation upon UV exposure and ∆Def1 ∆Rad6 mutants are more UV-sensitive than either individual mutant. This supports the notion that these proteins are part of competing pathways, repair and polymerase degradation, to rescue transcription from DNA damage.\cite{8,10} To test if the Rad26-Def1 complex antagonizes Def1 activity, I will utilize the rapamycin dimerization system to inducibly cross-link Rad26 and Def1 \textit{in-vivo}.\cite{11} If complex formation interferes with Def1-mediated Rpb1 degradation, I would expect addition of rapamycin to decrease Rpb1 polyubiquitination and degradation in response to UV damage. In contrast, when rapamycin is not added and the Rad26-Def1 complex is not forcibly induced, I would expect to see normal levels of Rpb1 degradation upon UV exposure. Rpb1 ubiquitination and degradation are quantifiable via western blot. Additionally, I will make point mutations at candidate residues in both Rad26 and Def1 and conduct \textit{in-vitro} binding experiments to identify and disrupt the binding interface of Rad26 and Def1, but not the proteins’ independent functions. I will express these mutants \textit{in-vivo} to test if disruption of the Rad26-Def1 complex promotes Rpb1 proteolysis in response to UV. If these experiments do not support an inhibitory function of the complex, I would explore other candidate proteins, such as the deubiquitinating enzyme Ubp3, that are posited to antagonize Def1 function\cite{12}. Taken together, these aims will help elucidate how Def1 is regulated both at the level of activation and by subsequent protein-protein interactions that may inhibit Def1 function. Understanding these regulatory steps will clarify the mechanism of how the cell chooses to degrade RNAPII in response to transcriptional stress.
Figure 1. Def1 localization in response to transcriptional stress. A. Unstressed conditions: Def1 shuttles between the nucleus and cytoplasm. The C-terminus of Def1 promotes cytosolic localization. Nuclear Def1 binds Rpb1. B. Transcriptional stress: Def1 is cleaved, driving Def1 accumulation in the nucleus.

Figure 2. Nuclear model of Def1 activation. 1. RNAPII arrests in response to transcriptional stress. 2. Rsp5 binds to stalled polymerase and ubiquitinates Rpb1 and Def1. 3. Ubiquitinated Def1 is proteolytically processed by the proteasome. 4. Cleaved Def1 recruits the ubiquitin ligase complex that ubiquitinates Rpb1, targeting it for degradation. The yellow dot indicates ubiquitin.

Figure 3. Cytoplasmic model of Def1 activation. 1. Transcriptional stress triggers Rsp5 to act on Def1. 2. Rsp5 ubiquitinates Def1. 3. Def1 is proteolytically cleaved by the proteasome, promoting nuclear localization. 4. Cleaved Def1 binds Rpb1 and recruits the ubiquitin ligase complex that ubiquitinates Rpb1, targeting it for degradation. The yellow dot indicates ubiquitin.

References