Published in final edited form as:

FEBS Lett. 2016 June ; 590(12): 1704–1712. doi:10.1002/1873-3468.12207.

Inhibition of yeast ribonucleotide reductase by Sml1 depends on the allosteric state of the enzyme

Tessianna A. Misko^{1,*}, Sanath R. Wijerathna^{1,*}, Tomas Radivoyevitch², Anthony J. Berdis³, Md. Faiz Ahmad¹, Michael E. Harris⁴, and Chris G. Dealwis¹

¹Department of Pharmacology, Case Western Reserve University, Cleveland, Ohio, 44106, USA

²Department of Quantitative Health Sciences, Cleveland Clinic Foundation, Cleveland, Ohio 44195, USA

³Department of Chemistry, Cleveland State University, Cleveland, Ohio, 44115, USA

⁴Department of Biochemistry, Case Western Reserve University, Cleveland, Ohio, 44106, USA

Abstract

Sml1 is an intrinsically disordered protein inhibitor of *Saccharomyces cerevisiae* ribonucleotide reductase (ScRR1), but its inhibition is poorly understood. RR reduces ribonucleoside diphosphates to their deoxy forms, and balances the nucleotide pool. Multiple turnover kinetics show that Sml1 inhibition of dGTP/ADP and ATP/CDP bound ScRR follows a mixed inhibition mechanism. However, Sml1 cooperatively binds to the ES complex in the dGTP/ADP form, whereas with ATP/CDP, Sml1 binds weakly and non-cooperatively. Gel filtration and mutagenesis studies indicate that Sml1 doesn't alter the multimerization equilibrium and the CXXC motif is not involved in the inhibition. The data suggest that Sml1 is an allosteric inhibitor.

Keywords

nucleotides; enzyme kinetics; oligomerization; mixed inhibition; intrinsically disordered protein

Ribonucleotide reductase (RR) catalyzes the conversion of ribonucleotides to 2'-deoxyribonucleotides, which is the rate determining step of dNTP synthesis. Allosteric regulation of RR substrate specificity and overall activity is essential for maintaining balanced and adequate deoxynucleotide pools during S-phase [1]. In other phases of the cell cycle, RR activity is also regulated at the level of transcription [2], subunit localization [3], and in the case of *Saccharomyces cerevisiae* RR (ScRR) and recently discovered in human RR (hRR), activity is limited by a small protein inhibitors, Sml1 (suppressor of mec-1

Correspondence to Chris G. Dealwis: Address: Case Western Reserve University, Wood Building 333, 2109 Adelbert Rd, Cleveland, Ohio 44106, USA, cxd114@case.edu, Phone: (216) 368-1652, Fax: (216) 368-1300.

*These authors contributed equally to this study. Authors are listed alphabetically.

Author Contributions

TAM, SRW, CGD, AJB, TR and MEH designed the work, and TAM and SRW performed the experimental work and analyzed the data. TR and AJB helped in the initial analysis of the kinetic data. MEH aided in the further analysis of the kinetic experiments. TAM, SRW, MEH, and CGD wrote the manuscript. MFA aided in sample preparation, intellectual discussion and critical comments on the manuscript.

lethality) and IRBIT, respectively [4–6]. Sml1 is believed to down-regulate ScRR activity through interactions with the catalytic subunit, but the mechanism by which it functions to block activity is unknown [4, 5, 7–10]. Here, we report multiple turnover inhibition kinetics and biochemical analyses of Sml1 that demonstrate a pattern of inhibition that depends on the identity of the allosteric regulator bound to the enzyme. Together the results suggest a mechanism in which effector binding modulates the affinity and cooperativity of Sml1, which may act to modulate down-regulation of ScRR activity during the cell cycle.

ScRR is representative of eukaryotic RR and consists of a multimeric catalytic α -subunit [$\alpha=n$ where $n=2, 4$ or 6] and a heterodimeric complex comprised of two β -subunits ($\beta\beta'$ heterodimers [$\beta\beta'=m$, $m=1, 2$ or 3], where the β' -subunit lacks the key residues required for generating the catalytic free radical) [11]. The α -subunit contains two different nucleotide binding sites that allosterically regulate substrate specificity and overall activity (Fig. 1A) [1, 12]. Binding of ATP, dATP, dGTP or dTTP to the allosteric specificity site (S-site) induces α -subunit dimers and determines the preference for NDP substrates binding to the catalytic site (C-site) where reduction of the 2'OH occurs (Fig. 1A) [1, 12–17]. A second allosteric site, termed the activity site (A-site), regulates the overall activity of the enzyme by binding either the allosteric activator ATP or the allosteric inhibitor dATP, eliciting the formation of either active or inactive RR α -subunit hexamers, respectively (Fig. 1A) [1, 12, 14–16, 18–21]. Both modes of regulation of RR are central to the maintenance of a balanced pool of dNTPs during DNA synthesis [18, 22].

ScRR activity is also under tight regulation by the small protein inhibitor Sml1. Sml1 is a 104-amino acid protein which is intrinsically disordered; and levels of Sml1 fluctuate throughout the cell cycle, diminishing when DNA synthesis or repair is needed [5, 7, 23, 24]. The cellular concentration of Sml1 is regulated by phosphorylation which leads to ubiquitination-dependent degradation [8, 23–29]. Sml1 is known to bind to the α -subunit, but little else is known regarding the mechanism by which it inhibits ScRR [4, 5, 7–10]. The ability of RR to exist in multiple allosterically regulated forms raises the question of whether these states have differential sensitivity to inhibition by Sml1.

Materials and Methods

Expression and Purification of Recombinant proteins

S. cerevisiae α -subunit (ScRR1) protein, $\beta\beta'$ -subunit (ScR2R4) and Sml1 were expressed and purified as described [15, 30, 31]. Yeast thioredoxin (TR) and thioredoxin reductase (TRR) were a gift from Dr. Sang Won Kang (Ewha Womans University, Seoul, Korea). Yeast TR and TRR protein were expressed and purified using the method adapted from Kim, J.A, *et al* [32]. CXXC deletion mutant was prepared using the forward primer- 5'-CAGAAGCTTGAGAAATGTGTTC-3' and reverse primer- 5'-GAACACATTTCTCAAGCTTCTG-3'. The expression, purification and biochemical characterization of CXXC ScRR1 is equivalent to that of wild type ScRR1.

Labeling of Mutant S60C Sml1

Sml1 containing a fluorescence tag was prepared as described in reference [33]. All fluorescent studies were done with Cys¹⁴Ser/Ser⁶⁰Cys labeled Sml1. Cys¹⁴Ser/Ser⁶⁰Cys Sml1 was labeled with Alexa 350 C₅-maleimide.

Size Exclusion Chromatography

Gel filtration was performed as described in references [16, 33]. All SEC work was carried out using a Superdex 200 10/300 GL column. ATP/dATP-induced oligomers of ScRR1 were analyzed in the presence of 3mM ATP/50μM dATP. The dATP hexamer-Sml1 complex was prepared by incubating 10μM of ScRR1 and 50μM dATP for 10 minutes and then by adding 40μM of Ser⁶⁰Cys Sml1 to a final volume of 100 μl. The resulting mixture was further incubated for 10 additional minutes on ice. For the ATP hexamer-Sml1 complex, we used 3mM ATP but used the same order of additions and incubation times as with the 50μM dATP hexamer complex.

Enzyme Assays

The steady state kinetics of ScRR were measured in the presence and absence of Sml1 *in vitro* using [³H]CDP and [¹⁴C]ADP reduction assays as described [16], using 0.5μM ScRR1 dimer and 5μM ScRR2•ScRR4 heterodimer. The radiolabeled dNDP products were separated from unreacted NDP substrates using borate chromatography and quantified by isotope counting [16]. When ATP/CDP was used as the effector-substrate pair, [³H]CDP concentrations were varied from 0.05 to 1mM. Similarly, when dGTP/ADP was used as the effector-substrate pair, [¹⁴C]ADP concentrations were varied from 0.05 to 1mM. ATP was omitted from dGTP/[¹⁴C]ADP experiments for the examination of ScRR dimer kinetics. For inhibition studies with [³H]CDP, 1, 1.5 and 2μM of Sml1 were used while in studies with dGTP/[¹⁴C]ADP 1, 2, and 3μM of Sml1 monomer were used, and with ATP/dGTP/[¹⁴C]ADP 0.2, 0.4, and 0.8 μM of Sml1 were used. All enzyme kinetic experiments were performed in duplicate.

Enzyme kinetic experiments performed in the presence of TR instead of DTT consisted of the same assay design as WT except for the substitution of the TR system (100μM TR, 1μM TRR, 2mM NADPH) in place of DTT. Prior to activity assay experiment, a dialysis of the ScRR1 sample was performed to remove DTT, which is needed for purification and storage purposes.

Analysis of steady state inhibition kinetics

To investigate the mechanism of Sml1 inhibition we evaluated steady-state ScRR reaction kinetics measured at a range of Sml1 and NDP substrate concentrations in the context of a general equilibrium model for mixed inhibition (Scheme 1).

To characterize the inhibition mechanism and estimate the apparent inhibitor affinity for E and ES and cooperativity of binding to these two enzymes forms, the steady state inhibition data were globally fit to a general rate equation for mixed inhibition (further description of derivation in supplemental text),

$$v_{\text{obs}} = \frac{[S]k_{\text{cat}}[E]_{\text{total}}}{K_m \left(1 + \frac{[I]^{n_E}}{K_i}\right) + [S] \left(1 + \frac{[I]^{n_{ES}}}{\alpha K_i}\right)}$$

in which K_i is the affinity of Sml1 to the free enzyme (E in Scheme 1) and αK_i is the affinity for the ES complex such that the value α measures the difference in affinity of Sml1 for these two enzyme forms. In Scheme 1, the parameter n is the stoichiometry of Sml1 binding to RR. In equation 1, n_E and n_{ES} are used as variables to evaluate cooperativity of binding of Sml1 to the free (E) and substrate-bound (ES) forms of the enzyme, respectively. The data were first fit to a model in which $\alpha=1$ to evaluate a purely non-competitive mechanism. However, a value less than one provided a better fit to the data. Models in which $n_E = n_{ES} = 1$ were subsequently evaluated and as discussed in the text, this model describes the inhibition mechanism of Sml1 inhibition of the ATP bound form of the enzyme. Integer values of n_E and n_{ES} greater than 1 were also individually tested for the dGTP bound form of the enzyme and the goodness of fit evaluated by comparing the magnitude of the squared differences between model and experimental data.

Results and Discussion

Steady state kinetic analysis of the modes of ScRR1 inhibition by Sml1

Dimerization of RR is induced by binding of the effectors dGTP, dTTP, dATP and ATP to the S-site, while binding of ATP to the A-site occurs with lower affinity and induces the formation of RR α -subunit hexamers [1, 13–17]. To gain information on the interaction of Sml1 with these different allosteric states, we first analyzed inhibition of the dimeric, dGTP-bound state of ScRR, which has specificity for ADP as a substrate. Linear fit of the steady state inhibition data on double reciprocal plots, $1/v_{\text{obs}}$ versus $1/[S]$, indicates that both the k_{cat}/K_m and k_{cat} are affected by the presence of Sml1, and the slope ($1/(k_{\text{cat}}/K_m)$) and y-intercept increase as a function of Sml1 concentration (Fig. 1B). This result demonstrates that Sml1 functionally interacts with both free enzyme (E) and the enzyme-substrate complex (ES). The double reciprocal plot also reveals that the decrease in k_{cat} (increase in $1/v_{\text{obs}}$ intercept) with increasing concentrations of Sml1 is non-linear, suggesting the binding of Sml1 to the ES complex is cooperative. Plots of the y-intercept ($1/k_{\text{cat}}$) vs Sml1 concentration illustrate the exponential dependence of the effect of inhibitor concentration on k_{cat} (Fig. 1E). In contrast, the interaction of Sml1 with free E appears to be non-cooperative. A plot of the slope ($1/(k_{\text{cat}}/K_m)$) vs Sml1 concentration shows linear dependence of k_{cat}/K_m on Sml1 concentration (Fig. 1F).

Global fitting of the steady state inhibition data to a general rate equation for mixed inhibition, as described in Materials and Methods, was used to evaluate different potential mechanistic possibilities for the dimeric, dGTP-bound state of ScRR (Fig. 1B inset, supplemental 1A). The simplest model consistent with the data involves non-cooperative binding of Sml1 to the free enzyme with a K_i of $0.8 \pm 0.12 \mu\text{M}$. Fitting the data, assuming tighter or weaker binding of Sml1 to ES relative to E ($\alpha < \text{or} > 1$), indicated that the data were best described by a mixed inhibition model in which Sml1 binds to ES with the tighter affinity ($\alpha K_i = 0.18 \pm 0.03 \mu\text{M}$) than E. Interestingly, the data are best described by a

mechanism assuming cooperativity of Sml1 binding to the ES complex ($n_{ES} = 2$). This observation is consistent with interpretations from inspection of the double reciprocal plot.

Next, we compared the mode of inhibition of the hexameric, ATP-bound form of ScRR by Sml1, which has specificity for CDP as a substrate. As shown by double reciprocal plots of the linear fit (Fig. 1C), Sml1 has a smaller effect on the k_{cat}/K_m and k_{cat} of this ATP bound allosteric state over the range of CDP concentrations tested, in comparison to the dimeric dGTP state. Unlike the dimeric dGTP-bound state of ScRR, a plot of the observed intercepts and slopes ($1/k_{cat}$ and $1/(k_{cat}/K_m)$) versus inhibitor concentration are both linear (Figs. 1E–F). This result is consistent with non-cooperative binding of Sml1 to both E and ES. Global fitting of these data to general rate equation reveals a ten-fold difference in binding affinity of Sml1 to E and ES, $20 \pm 5.6 \mu\text{M}$ and $2 \pm 0.59 \mu\text{M}$ respectively (Fig. 1C inset, supplemental 1B). These binding affinities are significantly lower relative to the dimeric dGTP bound ScRR. The substantial difference in the binding affinity to E and ES, with the apparent preference for ES indicates that Sml1 binding to the ATP/CDP bound ScRR also follows a mixed inhibition model. Most importantly, the global fitting further illustrated that the data are consistent with a model involving non-cooperative binding of Sml1 to both E and ES ($n_E = n_{ES} = 1$).

Two different patterns of ScRR inhibition by Sml1 are observed depending on if dGTP or ATP is bound in the S-site to allosterically regulate ScRR substrate specificity. However, these two reaction conditions also differ in the oligomerization state of the enzyme since ATP binding at the A-site induces hexamerization of RR α -subunit. To determine the extent to which oligomerization or the identity of the allosteric effector bound to the S-site correlate with the mode of Sml1 inhibition, we compared the inhibition of the hexameric dGTP-bound form in the presence of ATP to induce hexamer formation. Linear fit on a double reciprocal plot indicates visual similarity with the dimeric dGTP ScRR double reciprocal plot (Fig. 1D). Plot of the intercept vs Sml1 concentration shows there is an exponential dependence of the effect of inhibitor concentration on k_{cat} , indicating cooperative binding of Sml1 to ES (Fig. 1E). As also seen with both the dimeric dGTP and the hexameric ATP/CDP, interaction of Sml1 with E appears to be non-cooperative (Figure 1F). Global fitting analysis of the data for the ATP/dGTP-bound form of ScRR shows inhibition can be described by a mixed inhibition model in which Sml1 binds to E with a K_i of $0.18 \pm 0.03 \mu\text{M}$ and binds cooperatively ($n_{ES} = 2$) to ES with a αK_i of $0.07 \pm 0.01 \mu\text{M}$ (Fig. 1D inset, supplemental 1C). This analysis is consistent with expectations from inspection of the double reciprocal plot (Fig. 1D).

Examination of the inhibition kinetics of ScRR by Sml1 is relatively complex since it does not easily fit with a classic mechanism of inhibition: competitive, non-competitive or uncompetitive, but rather fits a mixed inhibition mechanism with specific aspects of this mechanism dependent on the allosteric state of the enzyme. Visual inspection of the double reciprocal plots, clearly shows that Sml1 does not exclusively act by competitively inhibiting ScRR. All allosteric states exhibit a k_{cat} change with increase in Sml1 concentration, indicating that Sml1 is not competing with the substrate, ADP or CDP, for binding at the C-site. It can also be argued that none of the allosteric states tested follow a purely non-competitive mechanism. Classical non-competitive mechanism exhibits equal affinity for E

and ES with no cooperativity. Attempts to fit this mechanism to the inhibition data proved to be unsuccessful. For the dimeric and hexameric ADP forms, the data fit a mixed inhibition model where there is a higher affinity for the ES than the E, 4.5 and 2.5 fold difference in affinity respectively. In the case of CDP, it can be said that it is a mixed inhibition mechanism since there is affinity for both the ES and E, but with greater affinity for ES (10 fold greater). Thus, an important shared attribute of all allosteric forms of ScRR is that there is a higher affinity for the ES than E. It is possible that the structural change in ScRR1 specifically induced by substrate binding allows for better exposure of the Sml1 binding site for a stronger binding affinity. The differences in the structure of ScRR1 when bound to either ADP or CDP might explain why different inhibition mechanisms are observed.

Sml1s effect on Oligomerization

The steady state inhibition supports functional binding of Sml1 to both E and ES in order to inhibit ScRR activity and the binding changes observed vary based on substrate and oligomerization states. So the question remains whether or not Sml1 was binding to the dimer or hexamer forms described in the kinetic assays as expected and whether inhibition by Sml1 binding can be further explained by changes in oligomerization. It is possible that the differences in binding to these different allosteric forms of the enzyme could reflect thermodynamic or mechanistic linkage between Sml1 binding and oligomerization. Therefore, size exclusion chromatography was used to test whether Sml1 forms stable complexes with dimeric and hexameric forms of ScRR α -subunit and examine its effect on oligomerization (Fig. 2). Fluorescently labeled Sml1 was incubated with ScRR hexamers formed in the presence of both dATP and ATP (Figs. 2A–B). Although it is not possible to maintain equilibrium conditions during chromatographic separation, the data clearly show that Sml1 forms stable complexes with ScRR dimers and hexamers (Fig. 2). Sml1 binds to both active (ATP bound) and inactive (dATP bound) hexamers (Figs. 2A–B) and does not appear to result in major alteration of the multimerization equilibrium under conditions used in enzyme assays (Figs. 2C–D).

Biochemical data are consistent with the functional association of Sml1 with both the free (E) and substrate bound (ES) forms of ScRR. Sml1 binding to the hexameric form of ScRR1 does not disrupt these hexamers, while Sml1 binding to the dimeric form does not shift the oligomerization equilibrium towards hexamer formation. Together this further supports the conclusions from the kinetic data, that binding and inhibition of ScRR1 by Sml1 is determined by allosteric state of ScRR, but the inhibition is not due to a shift in equilibrium of ScRR1 oligomeric state. The physiological role of Sml1, as supported by its stable binding to both ATP and dATP hexamers, can be attributed to complementing the allosteric inhibition of dATP. *In vitro* studies indicate dATP inhibits ScRR by 50% compared to ATP bound ScRR under physiological conditions, while the addition of Sml1 leads to the complete inhibition of ScRR (Supplemental Fig. 2)

Sml1 and the regeneration of the active site of ScRR1

It has been hypothesized that Sml1 might interfere with the reduction pathway in ScRR, targeting the CXXC motif located at the C-terminus of the α -subunit (ScRR1), an intermediate in the thioredoxin (TR) reduction pathway[9, 34]. In other words, does Sml1

inhibit ScRR by interfering with the rate limiting step of enzyme regeneration? After a catalytic cycle, the oxidized cysteines in the C-site require reduction prior to the next catalytic cycle. TR, one of the main reductants used in this process cannot directly reduce these cysteines so the intermediate, CXXC motif, is utilized. TR transfers a reducing equivalent to the CXXC motif and the flexible C-terminus tail then interacts with the cysteines in the C-site to transfer this reducing equivalent [34]. Removal of this CXXC motif renders the enzyme unable to be reduced naturally, but for experimental purposes, the synthetic two-electron reductant, DTT, can be used as the reductant, whose small size allows for the reduction of the active site directly [35]. To investigate the possibility that Sml1 is targeting the reduction pathway, the CXXC motif was removed and DTT was utilized. RR activity assay was utilized to measure the enzymatic activity levels of ScRR as determined by the amount of product formed, [^{14}C]ADP in this study. Measure of activity with WT and

CXXC mutant in the presence of DTT were used as controls to show that the mutant was functional and the results indicate that the CXXC mutant activity was similar to WT (Fig. 3). Activity in WT ScRR1 in the presence of Sml1 and either DTT or TX indicated that Sml1 can inhibit product production of ScRR1 despite which reductant is present. In both cases, there was essentially no product formation (Fig. 3). The key experimental condition however was the CXXC mutant in the presence of Sml1 and DTT. Two main possibilities were anticipated in this [^{14}C]ADP reduction experiment; either (1) there would be retention of activity with DTT and the mutant in the presence of Sml1, indicating Sml1 is targeting the CXXC motif, or (2) loss of activity, indicating that Sml1 is not targeting the CXXC motif but rather another site. Results indicate that there was no [^{14}C]ADP reduction with CXXC ScRR1 in the presence of DTT and 1 μM of Sml1 (Fig. 3).

Through this investigation of Sml1 targeting the CXXC motif at the C-terminus of ScRR1, we have observed that Sml1 inhibition is independent of this motif. While we can't rule out that Sml1 interferes elsewhere in the reduction pathway of ScRR1, it is important to know that Sml1 is not binding at the C-terminus. It is binding elsewhere on ScRR1 which might be causing a structural conformational change to prevent DTT mediated dNDP production. Without structural data of this complex, it is hard to distinguish precisely where Sml1 is binding on ScRR, eliciting either medium or long range interactions. Structural studies are currently being pursued to determine specifically how Sml1 is binding to inhibit ScRR activity.

Conclusions

Considering the importance of RR in maintaining an adequate and balanced cellular dNTP pool for the high fidelity of DNA replication and repair, the study of RR regulation has become a focus of attention for pharmacological intervention. The inhibition of budding yeast RR (ScRR) by Sml1 is one of the few examples of a natural protein RR inhibitor, making it a great research target for the development of future pharmacological interventions against cancer and other diseases characterized by rapidly dividing cells. One of the key characteristics of Sml1 that still remains a mystery is its mechanism of inhibition, which may help us structurally understand Sml1 as an intrinsically disordered protein and how it interacts with ScRR1. The data presented here are most consistent with a model in which Sml1 acts as an allosteric inhibitor of ScRR catalysis, exhibiting different affinity in the

presence and absence of effector-substrate pair and different mechanisms depending on the substrate bound (Fig. 4). It can be argued that the difference in binding between effector/substrate pairs seen here is simply because of the structural differences of ScRR bound to each effector/substrate.

It has been in debate since the discovery of Sml1 whether there is a human analogue of Sml1 and what the importance of studying Sml1 is. Recently there was the discovery of IRBIT, a proposed human analogue of Sml1 [6]. While the discovery of IRBIT is recent and there haven't been extensive studies on this inhibitor, there are some similarities between IRBIT and Sml1. Both IRBIT and Sml1 further decrease the activity of RR compared to just inhibition by dATP, with the distinction that IRBIT stabilizes only the dATP hRRM1 hexamer [6] and Sml1 has been shown to fully inhibit both the ATP and the dATP hexamer of ScRR. It was also noted that structurally, the N-terminal domain of IRBIT belongs to a class of IDPs (intrinsically disordered proteins) similar to Sml1 [6]. Similarly, p53R2 is known to make several protein-protein interactions of functional importance [36, 37]. The discovery of IRBIT validates the importance of studying Sml1 for the development of anti-proliferative therapeutics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank the present and past members of the Dealwis lab for contributing to the work of ribonucleotide reductase. We would also like to thank all of those in the RR and Sml1 field who have contributed so much work to the understanding of both of these fields. We would like to thank Dr. John J. Mieyal for the critical reading and comments on this manuscript. Funding was provided by the NIH [grant numbers R01GM100887, R01CA100827 and 5R25CA148052-05.]

Abbreviations

RR	ribonucleotide reductase
ScRR	<i>Saccharomyces cerevisiae</i> ribonucleotide reductase
SEC	size exclusion chromatography
dNTPs	deoxyribonucleotide triphosphates
NDPs	ribonucleoside 5'-diphosphates
dNDPs	deoxyribonucleoside 5'-diphosphates
C-site	Catalytic site
S-site	Specificity site
A-site	Activity site
ScRR1	α subunit of <i>Saccharomyces cerevisiae</i> ribonucleotide reductase

CXXC C-terminal motif

REFERENCES

1. Brown NC, Reichard P. Role of effector binding in allosteric control of ribonucleoside diphosphate reductase. *J Mol Biol.* 1969; 46:39–55. [PubMed: 4902212]
2. Huang M, Zhou Z, Elledge SJ. The DNA replication and damage checkpoint pathways induce transcription by inhibition of the Crt1 repressor. *Cell.* 1998; 94:595–605. [PubMed: 9741624]
3. Zhang Z, An X, Yang K, Perlstein DL, Hicks L, Kelleher N, Stubbe J, Huang M. Nuclear localization of the *Saccharomyces cerevisiae* ribonucleotide reductase small subunit requires a karyopherin and a WD40 repeat protein. *Proc Natl Acad Sci U S A.* 2006; 103:1422–1427. [PubMed: 16432237]
4. Chabes A, Domkin V, Thelander L. Yeast Sml1, a protein inhibitor of ribonucleotide reductase. *J Biol Chem.* 1999; 274:36679–3683. [PubMed: 10593972]
5. Zhao X, Chabes A, Domkin V, Thelander L, Rothstein R. The ribonucleotide reductase inhibitor Sml1 is a new target of the Mec1/Rad53 kinase cascade during growth and in response to DNA damage. *Embo J.* 2001; 20:3544–3553. [PubMed: 11432841]
6. Arnaoutov A, Dasso M. Enzyme regulation. IRBIT is a novel regulator of ribonucleotide reductase in higher eukaryotes. *Science.* 2014; 345:1512–1515. [PubMed: 25237103]
7. Zhao X, Muller EG, Rothstein R. A suppressor of two essential checkpoint genes identifies a novel protein that negatively affects dNTP pools. *Mol Cell.* 1998; 2:329–340. [PubMed: 9774971]
8. Zhao X, Georgieva B, Chabes A, Domkin V, Ippel JH, Schleucher J, Wijmenga S, Thelander L, Rothstein R. Mutational and structural analyses of the ribonucleotide reductase inhibitor Sml1 define its Rnr1 interaction domain whose inactivation allows suppression of mec1 and rad53 lethality. *Mol Cell Biol.* 2000; 20:9076–9083. [PubMed: 11074005]
9. Zhang Z, Yang K, Chen CC, Feser J, Huang M. Role of the C terminus of the ribonucleotide reductase large subunit in enzyme regeneration and its inhibition by Sml1. *Proc Natl Acad Sci U S A.* 2007; 104:2217–2222. [PubMed: 17277086]
10. Georgieva B, Zhao X, Rothstein R. Damage response and dNTP regulation: the interaction between ribonucleotide reductase and its inhibitor, Sml1. *Cold Spring Harb Symp Quant Biol.* 2000; 65:343–346. [PubMed: 12760048]
11. Perlstein DL, Ge J, Ortigosa AD, Robblee JH, Zhang Z, Huang M, Stubbe J. The active form of the *Saccharomyces cerevisiae* ribonucleotide reductase small subunit is a heterodimer in vitro and in vivo. *Biochemistry.* 2005; 44:15366–15377. [PubMed: 16285741]
12. Uhlin U, Eklund H. Structure of ribonucleotide reductase protein R1. *Nature.* 1994; 370:533–539. [PubMed: 8052308]
13. Eriksson M, Uhlin U, Ramaswamy S, Ekberg M, Regnstrom K, Sjoberg BM, Eklund H. Binding of allosteric effectors to ribonucleotide reductase protein R1: reduction of active-site cysteines promotes substrate binding. *Structure.* 1997; 5:1077–1092. [PubMed: 9309223]
14. Larsson KM, Jordan A, Eliasson R, Reichard P, Logan DT, Nordlund P. Structural mechanism of allosteric substrate specificity regulation in a ribonucleotide reductase. *Nat Struct Mol Biol.* 2004; 11:1142–1149. [PubMed: 15475969]
15. Xu H, Faber C, Uchiki T, Fairman JW, Racca J, Dealwis C. Structures of eukaryotic ribonucleotide reductase I provide insights into dNTP regulation. *Proc Natl Acad Sci U S A.* 2006; 103:4022–4027. [PubMed: 16537479]
16. Fairman JW, Wijerathna SR, Ahmad MF, Xu H, Nakano R, Jha S, Prendergast J, Welin RM, Flodin S, Roos A, Nordlund P, Li Z, Walz T, Dealwis CG. Structural basis for allosteric regulation of human ribonucleotide reductase by nucleotide-induced oligomerization. *Nat Struct Mol Biol.* 2011; 18:316–322. [PubMed: 21336276]
17. Zimanyi CM, Chen PY, Kang G, Funk MA, Drennan CL. Molecular basis for allosteric specificity regulation in class Ia ribonucleotide reductase from *Escherichia coli*. *eLife.* 2016; 5
18. Brown NC, Canellakis ZN, Lundin B, Reichard P, Thelander L. Ribonucleoside diphosphate reductase. Purification of the two subunits, proteins B1 and B2. *Eur J Biochem.* 1969; 9:561–573. [PubMed: 4896737]

19. Rofougaran R, Vodnala M, Hofer A. Enzymatically active mammalian ribonucleotide reductase exists primarily as an $\alpha_6\beta_2$ octamer. *J Biol Chem*. 2006; 281:27705–27711. [PubMed: 16861739]
20. Kashlan OB, Cooperman BS. Comprehensive model for allosteric regulation of mammalian ribonucleotide reductase: refinements and consequences. *Biochemistry*. 2003; 42:1696–1706. [PubMed: 12578384]
21. Wang J, Lohman GJ, Stubbe J. Enhanced subunit interactions with gemcitabine-5'-diphosphate inhibit ribonucleotide reductases. *Proc Natl Acad Sci U S A*. 2007; 104:14324–14329. [PubMed: 17726094]
22. Hakansson P, Hofer A, Thelander L. Regulation of mammalian ribonucleotide reduction and dNTP pools after DNA damage and in resting cells. *J Biol Chem*. 2006; 281:7834–7841. [PubMed: 16436374]
23. Andreson BL, Gupta A, Georgieva BP, Rothstein R. The ribonucleotide reductase inhibitor, Sml1, is sequentially phosphorylated, ubiquitinated and degraded in response to DNA damage. *Nucleic Acids Res*. 38:6490–6501. [PubMed: 20566477]
24. Uchiki T, Dice LT, Hettich RL, Dealwis C. Identification of phosphorylation sites on the yeast ribonucleotide reductase inhibitor Sml1. *J Biol Chem*. 2004; 279:11293–11303. [PubMed: 14684746]
25. Zhao X, Rothstein R. The Dun1 checkpoint kinase phosphorylates and regulates the ribonucleotide reductase inhibitor Sml1. *Proc Natl Acad Sci U S A*. 2002; 99:3746–3751. [PubMed: 11904430]
26. Uchiki T, Hettich R, Gupta V, Dealwis C. Characterization of monomeric and dimeric forms of recombinant Sml1p-histag protein by electrospray mass spectrometry. *Anal Biochem*. 2002; 301:35–48. [PubMed: 11811965]
27. Gupta V, Peterson CB, Dice LT, Uchiki T, Racca J, Guo JT, Xu Y, Hettich R, Zhao X, Rothstein R, Dealwis CG. Sml1p is a dimer in solution: characterization of denaturation and renaturation of recombinant Sml1p. *Biochemistry*. 2004; 43:8568–8578. [PubMed: 15222768]
28. Sharp JS, Guo JT, Uchiki T, Xu Y, Dealwis C, Hettich RL. Photochemical surface mapping of C14S-Sml1p for constrained computational modeling of protein structure. *Anal Biochem*. 2005; 340:201–212. [PubMed: 15840492]
29. Danielsson J, Liljedahl L, Barany-Wallje E, Sonderby P, Kristensen LH, Martinez-Yamout MA, Dyson HJ, Wright PE, Poulsen FM, Maler L, Graslund A, Kragelund BB. The intrinsically disordered RNR inhibitor Sml1 is a dynamic dimer. *Biochemistry*. 2008; 47:13428–13437. [PubMed: 19086274]
30. Chabes A, Domkin V, Larsson G, Liu A, Graslund A, Wijmenga S, Thelander L. Yeast ribonucleotide reductase has a heterodimeric iron-radical-containing subunit. *Proc Natl Acad Sci U S A*. 2000; 97:2474–2479. [PubMed: 10716984]
31. Nguyen HH, Ge J, Perlstein DL, Stubbe J. Purification of ribonucleotide reductase subunits Y1, Y2, Y3, and Y4 from yeast: Y4 plays a key role in diiron cluster assembly. *Proc Natl Acad Sci U S A*. 1999; 96:12339–12344. [PubMed: 10535923]
32. Kim JA, Park S, Kim K, Rhee SG, Kang SW. Activity assay of mammalian 2-cys peroxiredoxins using yeast thioredoxin reductase system. *Anal Biochem*. 2005; 338:216–223. [PubMed: 15745741]
33. SR W. Structural and Biochemical Studies of Ribonucleotide Reductase Inhibition by dATP and Sml1. Dissertation Thesis, CWRU. 2012:1–222.
34. Mao SS, Holler TP, Bollinger JM Jr, Yu GX, Johnston MI, Stubbe J. Interaction of C225SR1 mutant subunit of ribonucleotide reductase with R2 and nucleoside diphosphates: tales of a suicidal enzyme. *Biochemistry*. 1992; 31:9744–9751. [PubMed: 1390750]
35. Domkin V, Chabes A. Phosphines are ribonucleotide reductase reductants that act via C-terminal cysteines similar to thioredoxins and glutaredoxins. *Scientific reports*. 2014; 4:5539. [PubMed: 24986213]
36. Kuo M-L, Lee MB-E, Tang M, den Besten W, Hu S, Sweredoski MJ, Hess S, Chou C-M, Changou CA, Su M, Jia W, Su L, Yen Y. PYCR1 and PYCR2 Interact and Collaborate with RRM2B to Protect Cells from Overt Oxidative Stress. *Scientific reports*. 2016; 6:18846. [PubMed: 26733354]

37. Xie M, Yen Y, Owonikoko TK, Ramalingam SS, Khuri FR, Curran WJ, Doetsch PW, Deng X. Bcl2 induces DNA replication stress by inhibiting ribonucleotide reductase. *Cancer Res.* 2014; 74:212–223. [PubMed: 24197132]

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 1. Mode of inhibition of dimeric and hexameric ScRR by Sml1

(A) Structure of ScRR dimer with highlighted C-site, S-site and A-site. Structure of ScRR dimer (PDB 3S87) was generated in Pymol. (B) Linear fit of the dGTP/ADP dimer in a double reciprocal plot, demonstrating mixed inhibition. Inset is parameters provided from the global fit analysis. (C) Linear fit of the ATP/CDP hexamer in a double reciprocal plot, demonstrating uncompetitive inhibition. Inset is parameters provided from the global fit analysis. (D) Linear fit of the ATP/dGTP/ADP hexamer in a double reciprocal plot demonstrating mixed inhibition. Inset is parameters provided from the global fit analysis. (E) Intercept replot and (F) slope replot is also shown.

Figure 2. SEC analysis of Sml1-ScRR1 oligomerization

(A) Alexa 350 labeled Sml1 was used to monitor interactions with ScRR1 hexamer at 344 nm. C14S S60CSml1 bound to the dATP-induced ScRR1 hexamer was monitored at 344 nm (red trace). (B) Co-elution of Alexa 350 labeled Sml1 (red trace) with ATP-induced ScRR1 hexamer. (C) Injection of preformed dGTP ScRR1 dimer-Sml1 complex (blue line) to gel filtration column pre-equilibrated with 50 μ M dATP does not interfere with hexamer formation. Note, void volume of SEC column is 8 ml. (D) Injection of preformed dGTP ScRR1 dimer-Sml1 complex to gel filtration column does not induce hexamer formation. Note, void volume of SEC column is 8 ml.

Figure 3. Sml1 influence on the regeneration of ScRR1

Relative specific activities of wild type ScRR and CXXC ScRR in the presence and absence of Sml1 and in the presence of either DTT or the TR system. The CXXC ScRR1 in the presence of DTT as the reductant and Sml1 exhibits no activity, indicating that Sml1 does not inhibit the CXXC motif.

Figure 4. Models describing CDP and ADP inhibition

(A). Binding of Sml1 to dGTP/ADP bound ScRR1. Sml1 and substrate are indicated by (red circle) and (black dot) respectively. K_i and αK_i indicate the dissociation constant for the free enzyme and substrate bound enzyme respectively. States highlighted by the dotted box indicates cooperative binding of Sml1 to substrate bound enzyme. (B). Binding of Sml1 to ATP/CDP bound ScRR1. Sml1 and substrate are indicated by (red circle) and (black dot) respectively. K_i and αK_i indicate the dissociation constant for the free enzyme and substrate bound enzyme respectively. States highlighted by the dotted box indicates non-cooperative binding of Sml1 to substrate bound enzyme.

Scheme 1.