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The more the merrier: How homo-oligomerization alters the *interactome* and function of ribonucleotide reductase

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Abstract

Stereotyped as a nexus of dNTP synthesis, the dual-subunit enzyme—ribonucleotide reductase (RNR)—is coming into view as a paradigm of oligomerization and moonlighting behavior. In the present issue of “omics”, we discuss what makes the larger subunit of this enzyme (RNR- α) so interesting, highlighting its emerging cellular interactome based on its unique oligomeric dynamism that dictates its compartment-specific motives. Linking the history of the field with the multivariable nature of this exceedingly-sophisticated enzyme, we further discuss implications of new data pertaining to DNA-damage response, S-phase checkpoints, and ultimately tumor suppression. We hereby hope to provide ideas for those interested in these fields, and exemplify conceptual frameworks and tools that are useful to study RNR’s broader roles in biology.

New associations spawn new functions

Enzymologists, biochemists, and biologists have searched for paradigmatic systems to study protein-protein associations and accompanying interactomic changes. Despite there being so many examples, e.g., GAPDH[1] (Fig. 1a), aconitase[2] (Fig. 1b), and glcyl-tRNA synthetase[3] (Fig. 1c), in our opinion and arguably that of others, the protein ribonucleotide reductase (RNR), specifically the larger subunit, RNR- α , is one of the most diverse and intriguing[4,5]. There are many justifications for this assertion: few enzymes have brought together physics, chemistry, biology, cancer, and medicine like RNR[6–8]; furthermore, in few systems is the oligomerization equilibrium so rich. Finally, for few proteins are these equilibria so clearly linked to changes in interactomes and genomics, through complex feedback systems. Here we aim to discuss how RNR- α activity, interactome, locale, and function are regulated via RNR- α -specific oligomeric variations (Fig. 1d), and we link how those oligomerization shifts fit in with wider interactome and replisomic/genomic transitions occurring in cells in response to a range of stimuli.

RNR: the principal function

The principal biochemical function of RNR is dNTP generation for DNA synthesis during S-phase, and for DNA damage response (DDR) throughout the cell cycle[9]. RNR is a two-

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component enzyme, consisting of RNR- α and RNR- β (Fig. 2a). The latter is a smaller subunit present primarily in S-phase, where the RNR complex is canonically active, but degraded in G2/M. In the absence of RNR- β , p53R2 β , an isoform of RNR- β expressed constitutively at low levels (Fig. 2b), can provide dNTPs for DDR. All isoforms are predominantly cytosolic.

Cellular nucleotide pools are controlled through allosteric regulation of RNR- α , both substrate specificity and enzymatic activity[10]. Eukaryotic RNRs reduce diphosphates of all ribonucleosides (A, C, U, G), which are then converted to dNTPs (UDP is converted to dTDP as an intermediate step) (Fig. 2a). RNR- α serves as the nucleotide-binding subunit housing allosteric-activity and -specificity sites (A- and S-sites, respectively), beyond the catalytic/active site (C-site). The overall reductase-activity is controlled by the A-site: this functions like the gas pedal of a car, globally increasing reductase capacity when occupied by ATP, and globally downregulating activity when bound by dATP (Fig. 2c). However, not only are absolute levels of dNTPs, but relative levels of each dNTP, are also kept balanced by RNR. This regulation is performed through the S-site (Fig. 2c). For instance, binding of dGTP in the S-site stimulates RNR- α to reduce ADP.

Oligomeric transitions of RNR- α

The resting and active oligomeric states of RNR- α continue to be hotly debated (Fig. 3a). Under non-equilibrating conditions, in the absence or presence of all RNR- α -binding nucleotides *except dATP*, we and others have observed a monomer-dimer equilibrium, by gel filtration[11–14], and through FRET analysis under equilibrium conditions[14,15]. These findings contrast with results from other laboratories detailing higher-order oligomeric states during cycling and resting for RNR- α [16–18]. Such controversies are often found in biochemistry and likely relate to the varied methods used to analyse the different species and also how the contrasting analyses are carried out, in terms of, for instance, concentrations, presence or absence of other effector ligands and RNR- β , and buffers used, and whether experiments were executed under equilibrium versus non-equilibrium nature of the methods. What is clear from these studies is that whatever the resting state/cycling states of RNR- α -oligomers in the presence of specific native nucleotides are, the resulting multimeric species appear to be relatively transient and are not particularly stable.

Oligomeric regulation in the presence of dATP

Despite the largely conflicting results above, the hexamer-formation outcome achieved with nucleotide dATP is now universally accepted. Intriguingly, under non-equilibrium conditions, when RNR- α was treated with saturating dATP (i.e., a concentration that downregulates the enzyme-activity) and the resulting native protein was analysed by gel-filtration, only the typical monomer-dimer mixture was obtained[11,17]. However, when dATP was included in the running buffer of the gel-filtration, (a) hexamer(s) was(were) observed[11,17]. Thus, under saturating dATP, (a) hexameric state(s) is(are) formed. The X-ray structure of the yeast RNR- α -dATP-complex published around this time further substantiated these observations[17]. However, these data showed interesting quirks, as two

different RNR- α conformations were modeled[17]. It is difficult to understand the relevance of such pleomorphic arrangements, given that the conditions used for crystal growth are not physiological. Five years later, structural evidence for dATP-induced human hexamers was gleaned, using SAXS, X-ray, and EM analyses[19]. This work identified a single hexameric state, although it can be difficult to distinguish between hexamers with similar structures by many of the methods above. Another filamentous form of RNR- α was identified under “high” (10 mM dATP), although it remains to be known if this concentration is physiologically accessible as it would represent a state where dATP and ATP concentrations are equal[20]

Unfortunately, the poor stability of the dATP-induced hexamers rendered it difficult to assess if hexamers were formed in the cellular milieu. Furthermore, dATP cellular concentrations are difficult to assess accurately (and may well not be homogeneous in cells, rendering the 3–5 fold changes in dATP concentration upon treatment of cells with 0.3 mM dA, difficult to set fairly in context), and there is also some difficulty in defining the dATP affinity of RNR- α , as this molecule can occupy two binding sites on the protein (Fig. 2c). Thus, it is difficult to link in vitro affinities to in cell data with any confidence. dA is also extremely toxic to cells, further complicating cellular studies. What was really needed was dATP analogs that bind RNR- α rapidly (even in cells), have higher affinity than dATP, and are less acutely toxic than dATP. Studies on the approved leukemia drug, clofarabine (ClF), provided one such molecule. In vitro analysis showed that both ClFDP and ClFTP were reversible, but slow-release inhibitors of RNR- α , with defined affinities that were in the nanomolar range, at least 1000-fold more potent than dATP[11]. Experiments on susceptibility of the dATP-allosteric-regulation-defective mutant [RNR- α (D57N)] showed that ClFTP bound to the dATP binding site. However, ClFDP appeared to bind to the catalytic site, as may be expected of a (d)ADP analog. Surprisingly both these drugs elicited hexameric states of RNR- α , indicating that substrate analogs may induce (a) hexameric state(s)[11]. More intriguingly, these hexameric states persisted post drug dissociation[12]. Thus, it was likely that dATP and ClFTP/DP formed hexameric states, but the dATP-induced, inherently metastable, hexamers, and the persistent ClFTP/DP-induced hexamers were intrinsically structurally different in some way, further hinting at RNR- α 's ability to form pleomorphic hexameric states. Regardless of the intricacies, a dATP analog had been found that ushered (a) hexameric state(s) that is(are) persistent, and hence more ideal for cell culture experiments. Pleasingly, direct treatment of cells with ClF induced hexameric states, which were purified and shown to be hexameric by EM[12,21] (Fig. 3b). Although this does not prove that dATP can induce hexamers in cells, this finding demonstrates that RNR- α oligomeric equilibria are at play in cells and that what is seen in vitro is at least reflective of real biological phenomena. The fact that ClF is a clinically-used drug[22], meaning that RNR- α hexamers are likely present in treated patients, certainly helps to bring such relevance home. Finally, our subsequent investigations into behaviors of RNR- α in cells treated with different hexamer-inducing ligands relative to non-hexamer-inducing ligands, as outlined below, all point to the fact that dATP-induced hexamers and ClFTP/DP-induced hexamers are formed in cells and retain some of their properties we and others have described in vitro.

Ribonucleotide reductase forms pleomorphic hexameric states

Work from our laboratory with clinically-approved analogs of CIF, CLA and FLUMP (both of which inhibit recombinant RNR- α in the di- or triphosphate state) showed that all these drugs needed to downregulate RNR- α -dependent reductase activity for cytotoxicity[14]. These experiments relied on comparison of resistance induced upon expression of different RNR- α mutants. Overexpression of RNR- α (wt) was partially protective against toxicity of these drugs. However, overexpression of RNR- α (D57N), a mutant that cannot be inhibited by either CIF, CLA, or FLUMP as it is unable to form (an) inhibited hexameric state(s), but is as enzymatically-active as the wild-type, fully rescued toxicity[14]. Gemcitabine, an active-site binding mechanism-based irreversible inactivator of RNR- α - β -hetero-complex (for both wt and D57N mutant), toxicity was not fully rescued by overexpression of either protein-subunits[14]. These data once again hinted that hexamerization in cells was important.

We thus studied the hexameric state(s) formed by the three different dATP analogs by gel filtration and EM analyses among others [23] (Fig. 3c–d). Intriguingly, although there was overlap between the different structures formed, it was striking that the structures observed were pleomorphic. Further inspection showed that the different ligand-treated states formed structures with different symmetry. The RNR- α hexamers formed by the different drugs emerged to be differentially protease susceptible[14], indicating that there were structural differences between the different hexamers in solution.

Early examples of hexamer-specific interactomes

Based on this pleomorphism, and evidence of partitioning of structures, we postulated that the hexamers may help RNR- α gain new functions through changing RNR- α interactome(s). In 2014, a paper was published showing that at least some dATP-induced RNR- α hexamers bind to IRBIT[24], an ER-peripheral membrane protein. Intriguingly, this interaction promotes dATP binding to RNR- α , and hence increases the potency of dATP to downregulate RNR- α -supported reductase activity. Such an uncompetitive regulatory mechanism proffers an explanation for why RNR- α may undergo such drastic hexameric changes upon dATP binding, as there is a secondary recognition mechanism that assists dATP to regulate RNR- α activity.

This sort of regulation is also known to exist in other RNR- α s. For instance, Sml1/Spd1 are yeast proteins with no homolog in humans that directly inhibit RNR activity. However, in the case of Spd1/Sml1, this mode of regulation is orthogonal to dATP regulation, and is linked to DNA damage[25,26]. The Spd1/Sml1 pathway also does not need oligomeric regulation, or at least there is no firm link established. Regulatory differences between yeast and human RNR, and whether these disparate modes of regulation are linked, have not been systematically addressed. Thus, the discovery of IRBIT downregulating RNR-reductase activity[24], although an important moment in the RNR activity field, raised numerous questions to our minds: why does such a difference in regulatory modes between human and yeast proteins occur, especially when yeast RNR- α s can also form hexamers[17]? Are there other moving parts to this new signaling pathway in mammals/higher eukaryotes that require

it to be more intricate? Furthermore, the result does not really explain the ability or necessity, should one exist, to form pleomorphic hexamers.

Pleomorphic hexameric states lead to orthogonal interactomes

When our laboratory performed a Y2H-screen for RNR- α interactor(s)[23], one hit was the poorly characterized nuclear protein, ZRANB3, believed to be principally involved in regulating pathways chosen for DDR, in conjunction with ubiquitinated PCNA[27]. However, there were numerous aspects of ZRANB3 biology that were, at the time, unknown. ZRANB3 associates with PCNA in the absence of DNA damage, in so called replication factories, hinting at other roles outside DDR for ZRANB3, specifically in DNA replication, a process that RNR is heavily involved in.

We thus hypothesized that ZRANB3 and RNR- α can interact upon an unknown stimulus. Our first proposal was that RNR- α -hexamerization could be the stimulus. Such hexamerization is easy to achieve by treatment of cells with dA, or CIF/CLA/FLUMP[12,14], and we know the timescale for oligomerization states in cells[12], which proved to be a bonus. Early experiments showed that RNR- α indeed translocates into the nucleus, in the time taken to form the hexameric state upon treatment with dA or CIF[23]. This translocation requires importin- α 1. In the case of dA treatment, removal of dA leads to release of RNR- α from the nucleus on the time scale needed for excess dATP to be hydrolysed[23]. This is a critical piece of evidence showing that dATP-induced hexamers are grossly similar in vitro and in cells. More importantly, RNR- α inhibiting drugs that do not hexamerize RNR- α do not cause translocation, and the RNR- α (D57N) mutant (that does not hexamerize with dA/analogues) also does not translocate[23]. Based on our discussion on IRBIT above[24], we also postulated that IRBIT may negatively regulate RNR- α translocation; knockdown of IRBIT promotes RNR- α nuclear translocation and IRBIT overexpression inhibits nuclear translocation[23]. Subsequent experiments focused on understanding what the ZRANB3–RNR- α interaction could tell us. Intriguingly, RNR- α emerged to be an inhibitor of the basal ZRANB3–PCNA interaction (Fig. 4). Using several techniques, we showed that ZRANB3 is required to promote synthesis rate of the replisome (likely in association with PCNA), and that RNR- α is an inhibitor of this function[23] (Fig. 4).

Implications and outstanding questions underlying RNR-interactomes

Through these data, we begin to see a reason for the pleomorphic hexamers we and others have observed: RNR- α hexameric states must interact with several proteins, including at least Importin- α (that translocates RNR- α to the nucleus), IRBIT (the translocation inhibitor), and ZRANB3. This plasticity is thus put to good use in higher eukaryotes. However, this rationale still begs the question why does RNR- α hexamerization occur in organisms like yeast that lack ZRANB3 and do not have a clear equivalent to IRBIT or ZRANB3? Although it has not been studied extensively, it may be informative to ask how pleomorphic are the yeast and other lower eukaryotic RNR- α 's relative to their higher counterparts? In this way, we may start prising apart whether there was co-evolution of hexamer pleomorphism with DNA-synthesis regulation, or whether such regulation simply

highjacked a pre-existing pathway/proclivity of RNR- α that was adventitious. Furthermore, as RNR- α was found to bind a common, broadly-conserved PCNA-interacting domain[28], APIM (contained within 84 proteins in yeast), it is possible that RNR- α could regulate other aspects of the PCNA-interactome, either in yeast or mammals/yeast.

We also now know that ZRANB3 promotes rate of DNA-synthesis 2-fold in non-stressed cells[23,29–31]. Yet in ZRANB3 knockdown cells, DDR levels are unaffected[23]. It remains unknown how ZRANB3 functions in this context. It is clear that replication is itself inherently stressful and can induce DNA damage. It is thus possible that basal levels of DNA damage, that are incurred during S-phase, are fixed by ZRANB3 in normal cells, but these lesions remain unfixed, slowing down DNA-synthesis when ZRANB3 is absent. However, such an explanation does not explain why DDR is not upregulated in ZRANB3-knockdown cells. Furthermore, RNR- α is not able to outcompete binding of ZRANB3 to ubiquitinated-PCNA (which binds more strongly to ZRANB3 than non-ubiquitinated PCNA), and PCNA-ubiquitination is not affected by RNR- α -overexpression[23], so this hypothesis cannot explain the effects of nuclear RNR- α either. Such data further hint that ZRANB3-mediated DNA-synthesis promotion could also be impacted in the DNA-damaged state, offering the possibility that ZRANB3 may act like a switch, regulating DNA synthesis and focusing resources to fix breaks[32]. Indeed, how DNA synthesis and origin firing are regulated during DNA damage during the “S-phase checkpoint” remain an area of significant debate and it would be most interesting to investigate this intersection[33]. Intriguingly, a protein related to ZRANB3, SMARCAL1[34], travels down replication forks, in the absence of DNA damage, hinting that SMARCAL1 may be a substoichiometric component of the replisome and *could* have similar properties to ZRANB3. The differences in regulation of these two similar proteins could serve as important comparisons to understand the function and regulatory processes involved in DNA synthesis, and we will outline some of the differences recently discovered below.

For many years it has been known that RNR- α suppresses Ras induced transformation[5]. Indeed, the preponderance of evidence points to the fact that RNR- α is a haplo-insufficient tumor suppressor[5,23]. Exploring this link, and the intersection with the replisome through ZRANB3 regulation is one of the most interesting avenues for future study, and could open new doors to therapeutic design and development. Indeed, new data are starting to bring this question better into focus. It appears that ZRANB3 is required for DNA synthesis, particularly under conditions of high DNA synthesis stress (such as myc upregulation[35]). Loss of ZRANB3 activity (even if one ZRANB3 allele remains) strongly suppresses myc- (an oncogene that cooperates with Ras[36]) induced tumorigenesis and prolongs lifespan in mouse models of B-cell lymphoma. Extension of this logic explains why nuclear RNR- α has similar properties (it is a ZRANB3 inhibitor). Most intriguingly, SMARCAL1, mentioned above, shows similar traits to ZRANB3 reduction in the double knockout, but in the heterozygote serves to promote tumorigenesis. The divergent behaviors of such similar proteins imply that ZRANB3's function is in some way additive to oncogenes, and could explain why RNR- α has evolved ZRANB3 affinity.

In sum, although the biology of the fascinating pair of enzymes that make up RNR is starting to be unravelled, we have a long way to go, even with the larger subunit. We feel

that the field is ripe for more development and for further investigation and encourage researchers to take up the baton in this area. We have developed many plasmids, mutants, and lines that can be of use in these studies, and there are many more available from others. These constructs together with our specific RNR- α -perturbing reagents/protocols are ideal for studying specific aspects of the inherently plastic RNR- α interactome. We believe that such studies will illuminate that RNR- α regulates essential, and indeed ancient apparatus beyond the replisome.

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Abbreviation List

CLA	cladribine
CIF	clofarabine
DDR	DNA damage response
FLUMP	fludarabine monophosphate

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Figure 1: Canonical and moonlighting functions of representative enzymes.

a) Tetrameric GAPDH performs its canonical function catalyzing oxidative phosphorylation of glyceraldehyde-3-phosphate in the cytosol. The monomeric form plays a moonlighting role in the nucleus, converting uracil to cytosine. PDB ID human GAPDH: 4WNC.

b) Holo-aconitase (Aco) in iron-rich (normal) conditions is bound to a $[4\text{Fe-4S}]$ cluster and interconverts citrate and isocitrate as part of the citric acid cycle. In iron-deficient conditions, holo-aconitase loses the $[4\text{Fe-4S}]$ cluster and undergoes structural changes, losing its enzymatic ability. The resultant apo form binds mRNA transcripts housing iron-response elements (IREs). Depending on the position of the IRE relative to the coding region (3' or 5'), the apo-aconitase binding can either block degradation or ribosomal binding, respectively. TCA, tricarboxylic acid. Grey dots and cube respectively illustrate free ions and the bound $[4\text{Fe-4S}]$ cluster.

c) Glycyl-tRNA synthetase (GlyRS) canonically charges tRNA^{Gly} with glycine, a process essential for protein synthesis. GlyRS also participates in controlling global neddylation levels by interacting with various components within the neddylation-dependent cell cycle.

signaling pathway for proliferation and survival. GlyRS directly interacts with NEDD8, a ubiquitin-like protein, and activated (neddylated) Ubc12 (an E2 enzyme in the neddylation pathway). This process ultimately leads to activation of cullin proteins through neddylation, allowing the cell to progress through the cell cycle. PDB ID: human GlyRS 2ZT5.

d) RNR- α forms a heterotetramer with RNR- β or RNR-p53 β . The resulting complex constitutes the minimally-required quaternary state for reductase activity that catalyzes the reduction of NDPs (N=A,U,G,C) to dNDPs. RNR catalysis is the only means available for de novo dNTP synthesis required for DNA replication and repair. Binding of the di- and/or triphosphates of the indicated compounds to RNR- α results in formation of hexameric states, ultimately leading to anti-proliferation effects. PDB IDs: Human RNR- α dimer: 3HND, human RNR- α hexamer: 6AUI. Ribbon structure of heterotetramer is a model for an enzymatically active RNR-complex derived from *E. coli* RNR-subunits: 4R1R, 3R1R, 1R1B.

Figure 2: Canonical enzymatic function of RNR- α is tightly regulated by expression of partner subunits RNR- β and various allosteric effectors.

a) Heterotetramer of $\alpha_2\beta_2/p53\beta_2$, the minimal quaternary state required for reductase activity, enables NDP reduction dNDPs (N=A,U,G,C), which are then converted to dNTPs by kinases (with dUDP undergoing additional transformations, beyond phosphorylation, prior to formation of dTTP). Ribbon structure of heterotetramer is a model for an enzymatically active RNR-complex derived from *E. coli* RNR-subunits: 4R1R, 3R1R, 1R1B.

b) Schematic illustrating constitutive expression of RNR- α protein independent of the cell-cycle stages; primarily S-phase-specific expression of RNR- β ; and constitutive yet low expression levels of RNR-p53 β .

c) Crystal structure of human RNR- α dimer with TTP effector and GDP substrate bound to S- and C-sites, respectively (PDB ID: 3HND). In the reductase-active heterotetrameric state ($\alpha_2\beta_2$) (see Fig. 2a), the C-site functions as catalytic site and binds NDP substrates. The A-site (allosteric activity site) functions as the gas pedal of the enzyme: binding of ATP and dATP respectively up- or downregulates reductase activity. The S-site (allosteric specificity site) serves as the gear-shifter of the system: binding of dGTP, dATP/ATP, dTTP and dATP/ATP at S-site respectively selects for ADP, UDP, GDP, CDP, to be reduced at C-site.

Figure 3: Resting and active oligomeric states of RNR- α in cells modulated by various substrates/ effectors remain under debate but dATP-induced hexamer-formation is universally accepted.

a) Factors/ligands controlling the oligomeric equilibrium of RNR- α in cells (i.e., interconversions between the three key states: monomer, dimer, and hexamer) remain largely unclear, except for dATP and clinically-used nucleotides, where hexamerization is demonstrated in cells and in vitro.

b) Top: Electron microscopy (EM) of RNR- α complexes isolated from cells stimulated with CIF. Bottom: EM image of recombinant His₆-RNR- α treated with CIFDP. Images reproduced from Aye et al, 2012 Cell Chem Bio[12].

c) Gel filtration analyses of recombinant human RNR- α with and without pre-treatment with hexamer-inducers demonstrate the existence of hexameric states. With untreated protein, a monomer is observed (green trace). Protein pretreated with hexamer-inducer nucleotide (here, FLUDP) and subsequent gel filtration analysis (in the running buffer containing no nucleotide), shows a different elution profile corresponding to hexameric states (dotted dark trace).

d) EM analyses of hexamer-inducer-treated recombinant human RNR- α support the existence of hexameric states. The hexameric states, those induced by the same ligand and different ligands (shown here for the three anti-leukemic drugs), display disparate symmetries and conformational heterogeneity, which is further supported by independent biochemical assays. Cryo-EM image reproduced from Fu et al, Nature Chem Bio 2018[22].

Figure 4: Depiction of canonical and moonlighting functions of RNR- α .

Certain hexameric conformation(s) of RNR- α induced by dATP or nucleotide drugs result(s) in nuclear import, assisted by importin- α 1 and negatively regulated by cytosolic binder, IRBIT. Inside the nucleus, RNR- α (irrespective of dimeric and/or hexameric states) directly interacts with ZRANB3 (in the process antagonizing ZRANB3 binding to PCNA [which may occur as 3:1 ZRANB3:PCNA[37], leading to DNA-synthesis suppression and thus growth inhibition. *Inset:* Canonical behavior of RNR- α promotes DNA-synthesis, hence cell-proliferation. PBD IDS; human PCNA: 6FCM, human Importin- α : 4B18, human IRBIT: 3MTG, human RNR- α dimer: 3HND. Ribbon structure of heterotetramer is a model for an enzymatically active RNR-complex derived from *E. coli* RNR-subunits: 4R1R, 3R1R, 1R1B.