ATM-mediated serine 72 phosphorylation stabilizes ribonucleotide reductase small subunit p53R2 protein against MDM2 to DNA damage

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Ribonucleotide reductase small subunit p53R2 was identified as a p53 target gene that provides dNTP for DNA damage repair. However, the slow transcriptional induction of p53R2 in RNA may not be rapid enough for prompt DNA damage repair, which has to occur within a few hours of damage. Here, we demonstrate that p53R2 becomes rapidly phosphorylated at Ser72 by ataxia telangiectasia mutated (ATM) within 30 min after genotoxic stress. p53R2, as well as its heterodimeric partner RRM1, are associated with ATM in vivo. Mutational studies further indicate that ATM-mediated Ser72 phosphorylation is essential for maintaining p53R2 protein stability and conferring resistance to DNA damage. The mutation of Ser72 on p53R2 to alanine results in the hyperubiquitination of p53R2 and reduces p53R2 stability. MDM2, a ubiquitin ligase for p53, interacts and facilitates ubiquitination of the S72A-p53R2 mutant more efficiently than WT-p53R2 after DNA damage in vivo.

Our results strongly suggest a novel mechanism for the regulation of p53R2 activity via ATM-mediated phosphorylation at Ser72 and MDM2-dependent turnover of p53R2 dephosphorylated at the same residue.

DNA damage stress | protein stability | signal transduction | kinase

DNA is surprisingly reactive and is under continuous assault from daily environmental agents such as UV light, reactive chemicals, and metabolic byproducts such as reactive oxygen species. Defects in DNA damage signaling and repair can lead to mutations, ultimately resulting in cancer (1). In addition to a potential role in cancer development, damage to cellular DNA has been used for cancer therapy and is responsible for most of the toxic effects of such therapy (1, 2). Therefore, the study of genes involved in DNA damage responses could lead to a deeper understanding of cancer development and more effective treatments of malignancies.

Ribonucleotide reductase (RR) is a rate-limiting enzyme responsible for providing a balanced dNTP supply for DNA synthesis and repair (3). Unbalanced dNTP supply can lead to genetic abnormalities and cell death, underscoring the importance of the mechanisms that regulate RR activity. RR is composed of two nonidentical subunits, RRM1 and RRM2. p53R2, an analogue of RRM2 in mammalian cells, can substitute for RRM2 to interact with RRM1 and plays an important role in DNA damage induced by genotoxic stress (4, 5). p53R2 has been identified as a transcriptional target of p53 (4), whereas RRM2 is transcriptionally regulated by cell cycle associated factors, such as nuclear factor Y and E2F (6). However, the mechanism by which p53R2 activity is induced by p53 may not be rapid enough to supply dNTPs for prompt DNA repair, which can be completed within a few hours after DNA damage (1, 3). More than 90% of damaged DNA can be repaired within 8 h after non-lethal UV irradiation in HeLa cells. Yet, the p53R2 mRNA is not fully induced until 12 h after γ-irradiation in many of p53 WT cells including normal dermal fibroblast, MCF-7, LoVo, and HCT116 cell lines (4). Additionally, we have previ-
Ser72 phosphorylation of p53R2, as well as Ser1981 phosphorylation of ATM induced by cisplatin, UV radiation, and ionizing radiation (Fig. 2C). Moreover, polyubiquitination of both mutations (S72A/S112A-p53R2) did not result in any further reduction of phosphorylation compared with the S72A-p53R2 mutant (Fig. 1C).

Ser 72 Phosphorylation by ATM Stabilizes p53R2 Protein Against UV Irradiation. To confirm whether p53R2 is phosphorylated at Ser72 in cells following genotoxic stress, a polyclonal antibody against phosphorylated Ser72 of p53R2 (anti-pS72-p53R2) was generated. The anti-pS72-p53R2 antibody recognized WT-p53R2, but not the S72A-p53R2 mutant, after co-transfection with ATM plasmids. WT-p53R2 alone, without co-transfection with ATM plasmids, was not detected by this antibody (Fig. 2A). Moreover, this antibody also detected an endogenous phosphorylated form (pS72A-p53R2 mutant transiently transfected in 293 cells also underwent UV-induced protein degradation, whereas WT-p53R2 was stable (Fig. 2D). Similar results were obtained in pulse-chase experiments (Fig. 2E). UV induced rapid decay of S72A-p53R2 (Fig. 2E), but failed to alter the amount of radiolabeled WT-p53R2 (Fig. 2E). p53R2 degradation induced by UV in A-T cells can be blocked in cells pretreated with the proteasome inhibitor MG-132 (Fig. S2), suggesting that UV-induced S72A-p53R2 decay is through the proteasomal pathway. Interestingly, polyubiquitinated forms of the S72A-p53R2 mutant were detected, but little WT-p53R2 ubiquitination was seen after co-expression with a ubiquitin expression plasmid in 293 cells following UV exposure (Fig. 2F).

MDM2 Interacts with and Facilitates Ubiquitination of S72A Mutant p53R2 In Vivo. MDM2, an E3 ubiquitin ligase that targets p53 for proteasomal degradation, interacts with p53 dynamically in response to UV irradiation (11). Given that p53R2 is a newly identified ATM substrate, we next tested whether MDM2 is involved in UV-induced ubiquitination and degradation of unphosphorylated p53R2. In transient transfection experiments, we found that co-expressing MDM2 promoted UV-induced polyubiquitination of S72A-p53R2 that was seen only in cells treated with the proteasome inhibitor MG132 (Fig. 3A). To further investigate if MDM2 contributes to the degradation of p53R2, we examined endogenous p53R2 levels in MDM2-knockout mouse embryonic fibroblasts (MEFs) (12) in which ATM proteins were knocked down by siRNA. As shown in Fig. S3, p53R2 accumulated more in p53−/−/MDM2−/− MEFs than in p53−/− MEFs after UV irradiation. To further characterize the functional interaction between MDM2 and p53R2, we examined whether these two proteins interact. We first examined the interaction between endogenous p53R2 and MDM2 (Fig. 3B). Using co-immunoprecipitation (co-IP) assays, we observed that MDM2 was present in the immunocomplexes of p53R2 before UV treatment (Fig. 3B). The interaction between MDM2 and p53R2 was disrupted in ATM proficient cells (Y7S) at later time points after UV exposure (Fig. 3B, Left). In contrast, endogenous p53R2 in ATM-deficient cells (pEB7) remained associated with MDM2 after UV exposure (Fig. 3B, Right). This interaction was also detected between exogenously expressed MDM2 with WT-p53R2 or S72A-p53R2 in 293 cells before exposure to UV (Fig. 3C). However, lower levels of MDM2 proteins dissociated from S72A mutant p53R2 compared with WT-p53R2 proteins following UV exposure. These results suggest that hyperubiquitination and degradation of S72A mutant p53R2 might result from a higher binding affinity with MDM2.

p53R2 and RRM1 Associate with ATM. To demonstrate a physical interaction between ATM and p53R2, we performed co-IP experiments. In support of the aforementioned observation that ATM is a direct p53R2 kinase, endogenous ATM and p53R2 were co-immunoprecipitated in UV-irradiated cells (Fig. 4A). ATM was brought down by p53R2 specific antibodies as early as 15 min after UV irradiation (Fig. 4A). This interaction was further verified by a reciprocal co-IP assay using ATM-specific antibody. However, when ATM antibody was used, p53R2 was already associated with ATM before UV irradiation (Fig. 4B). Their interaction was clearly enhanced around 30 min after exposure to UV. In addition to p53R2 polypeptide, RRM1 (which has a molecular mass of 80 kDa) was also detected in the ATM immunoprecipitate (Fig. 4C). These results suggested that RRM1 and p53R2 can form a complex with ATM in vivo. However, p53R2 was not detected in ATR (Fig. 4C) or DNA-PKcs immunocomplexes (Fig. 4D).

S72A-p53R2 Exerts a Dominant-Negative Effect in Cell Cycle Progression and Increases Cellular Sensitivity to DNA Damage Stress. We next evaluated the effect of p53R2 phosphorylation on cell cycle progression following DNA damage. Stable cell lines overexpressing...
pressing Myc-tagged WT-, S72A-p53R2, or vector control were generated. Expression of the transgenes was assessed by immunoblotting analysis using a Myc-tagged specific antibody (Fig. S4A). All cell lines had a normal cell cycle distribution before UV exposure (Fig. 5A). Following UV exposure, WT-p53R2 or vector control expressing cells exhibited a time-dependent decrease in G1 phase accompanied by an increase in S phase cells (0, 3, and 12 h) and a later increase in cells in G2/M phase (24 and 36 h). UV-induced G2/M arrested cells were slightly increased in WT-p53R2 transfected cells compared with vector transfected cells. However, such G2/M phase accumulation was not detected in S72A-p53R2 expressing cells. Instead, these cells accumulated predominantly in the S phase and decreased in the G1 phase. Most of the S72A-p53R2 expressing cells failed to enter the G2/M phase (Fig. 5A). We also monitored the dynamic response of these cells to UV under an RT-CES system. There was no difference in cell growth among these cells without UV treatment (Fig. S4B). However, S72A-p53R2 expressing cells resulted in a delayed growth curve compared with WT-p53R2 expressing cells, whereas vector control expressing cells had a growth curve between WT- and S72A-p53R2 expressing cells (Fig. S4B).

Aberrant cell cycle progression may trigger a cell death response. We next used MTT assay to analyze the viability of those cells after exposure to genotoxic stress, including UV, IR, and cisplatin. As expected, S72A-p53R2 expressing cells are the most susceptible to all three genotoxic treatments (Fig. 5B). Cells overexpressing p53R2 with a mutation at Ser112 had a genotoxic sensitivity similar to that in cells expressing WT- or S72A-p53R2, or empty vector. After 24 h, cells were exposed to UV and ubiquitination was examined by immunoprecipitation with anti-Myc antibodies and immunoblotting with HA antibodies.

Discussion

Our results demonstrate that ATM phosphorylates p53R2 at Ser72 in response to genotoxic stress and that this modification is essential for maintaining p53R2 protein stability. Mutation of Ser72 to alanine rendered p53R2 both hyperubiquitinated and...
less stable than WT-p53R2 after UV irradiation. The role of ATM signaling has been implicated in the regulation of p53R2 protein expression (13), but the underlying mechanism has not been clear. Here we present direct evidence that ATM not only phosphorylates but also interacts with p53R2. The phosphorylation of p53R2 at Ser72 occurs at a time point that corresponds to the enhanced binding between p53R2 and ATM. The timing of phosphorylation at this site is also closely linked to the change in stability of p53R2 in A-T cells following UV irradiation. Therefore, we hypothesized that ATM-dependent phosphorylation at Ser72 in p53R2 potentially contributes to its stability against UV induced degradation.

Unlike RRM2, p53R2 does not contain a KEN box sequence, which can be targeted by the Cdh1-APC E3 complex for proteolysis during G0/G1 arrest and late mitosis (14, 15). However, we observed that endogenous p53R2 in A-T cells or S72A-p53R2 expressed in 293 cells was sensitive to UV exposure (Fig. 2) even though their mRNA levels remained unchanged (data not shown). Here we demonstrate that MDM2 promotes ubiquitination and degradation of S72A-p53R2. p53R2 protein accumulates in MDM2-deficient and ATM knockdown cells after UV exposure (Fig. S3). Our data suggest that hyperubiquitination of p53R2, were preincubated with MG-132 for 30 min before exposure to UV (20 J/m²). At the indicated times, cells were lysed and immunoprecipitated with Myc-tagged antibody, followed by immunoblotting with MDM2 or Myc-tagged antibodies.

**Fig. 3.** MDM2 ubiquitinates and interacts with p53R2. (A) MDM2 induced S72A mutant p53R2 ubiquitination in response to UV. 293 cells were transiently transfected with the plasmids encoding Myc-tagged WT- or S72A-p53R2 along with ATM and MDM2. The cells were preincubated with or without MG-132 for 30 min, followed by stimulation with or without UV (20 J/m²) for 6 h. After treatment with UV, the cell lysates were immunoprecipitated with anti-Myc. Ubiquitination of WT- or S72A-p53R2 was examined by immunoblotting with a specific ubiquitin antibody. p53R2 protein levels were examined by anti-p53R2 antibody. (B) Enhanced interaction between endogenous MDM2 and unphosphorylated p53R2. Cell lysates isolated either from pEB57-Y75 (ATM proficient) or MG132-pretreated pEB57 (ATM deficient) cells at the indicated times after UV stress (20 J/m²) were immunoprecipitated with anti-ATM antibody, followed by immunoblotting with anti-DDK- or anti-p53R2 antibodies. (C) Enhanced interaction between exogenous MDM2 and S72A-p53R2 mutant. 293 cells, after transient transfection with plasmids encoding Myc-tagged WT- or S72A-p53R2 together with ATM and MDM2, were preincubated with MG-132 for 30 min before exposure to UV (20 J/m²). At the indicated times, cells were lysed and immunoprecipitated with Myc-tagged antibody, followed by immunoblotting with MDM2 or Myc-tagged antibodies.

**Fig. 4.** Interaction between ATM and p53R2. (A) Co-IP of endogenous ATM by anti-p53R2 antibody. Cell lysates were isolated from KB cells at the indicated times after UV stress (20 J/m²) and immunoprecipitated by anti-p53R2 antibody, followed by immunoblotting with anti-ATM or anti-p53R2 antibodies. (B) Same as in A except that ATM antibody was used for co-IP, followed by immunoblotting with anti-ATM, anti-p53R2, and anti-RRM1 antibodies. (C) Same as in A except that ATR antibody was used for co-IP, followed by immunoblotting with anti-ATR or anti-p53R2 antibodies. (D) Same as in A except that DNA-PKcs antibody was used for co-IP, followed by immunoblotting with anti-DNA-PKcs or anti-p53R2 antibodies.
uitination and degradation of S72A mutant p53R2 after UV stress might be a result of its strong binding with MDM2 (Fig. 3 B and C). MDM2 is well known for its role as a p53 ubiquitin ligase (11). We found that p53R2, p53, and ATM can form a trimeric protein complex (Fig. S5), but whether p53R2 and p53 compete against each other as targets for MDM2 ubiquitination remains to be investigated.

p53R2 has been implicated in the regulation of cell cycle progression induced by stress from DNA damage. p53R2-null cells or cells treated with the RR inhibitor hydroxyurea prevented G2/M arrest and resulted in cell death (4). Similarly, cells overexpressing S72A-p53R2 exhibited abnormal cell cycle progression and enhanced sensitivity to genotoxic stress (Fig. 5). As demonstrated in Fig. 4, p53R2 constitutively interacts with RRM1. Therefore, overexpressed S72A-p53R2 might out-compete WT-p53R2 in binding RRM1 and disturb cell cycle progression. Measuring RR activity by the quantification of total dNTP revealed that overexpressed S72A-p53R2 reduced basal and UV-induced dNTP levels compared with WT-p53R2 and mock vector (Fig. S6).

Recent studies demonstrate that ATM regulates the function of p53R2 by providing dNTP for mitochondrial DNA synthesis (13, 16). However, there seems to be no significant difference in the content of mtDNA in cells overexpressing WT- or S72A-p53R2 before or after UV exposure (Fig. S4C). We previously identified a potential role of p53R2 in scavenging reactive oxygen species (ROS) and protecting mitochondrial membrane potential against oxidative-stress-induced damage (17). It will be of interest to determine whether the enhanced sensitivity of S72A-p53R2 expressing cells to genotoxic stress results from abnormal accumulation of reactive oxygen species.

In this study we demonstrate a direct link between p53R2 and ATM. Cells deficient either in ATM or p53R2 exhibit defects in DNA damage repair and cell cycle progression. p53R2 and RRM1 are normally expressed at low basal levels in resting cells, and both proteins can be translocated into the nucleus immediately upon genotoxic stress (4, 5). Interestingly, p53R2 is detected in the MRE 11 complex (NBS1/Rad50/MRE11; Fig. S7), a DNA damage complex involved in double-strand break repair and DNA damage checkpoint pathways (18), further suggesting that p53R2 is part of the DNA damage complex. Similar to NBS1, p53R2 can pre-associate with ATM before UV stress (19). Their interaction is enhanced after UV exposure. The increase in complexed ATM and p53R2 might be the result of conformational changes induced in the protein complex upon DNA damage. Recent evidence demonstrated that ATM at site-specific DSBs requires functional NBS1 protein, ATM kinase activity, and auto-phosphorylation of ATM at Ser 1981 (20). Therefore, ATM may immediately recruit the pre-associated RRM1 and p53R2 proteins along with the MRE11 complex to the DNA damage sites to supply dNTP during the acute phase of DNA damage repair. Weak or indirect interac-
tions might explain why binding between p53R2 and ATR or DNA-PKcs was not observed (Fig. 4 C and D). Interestingly, ATR’s binding partner ATR-interacting protein (21), but not DNA-PKcs regulatory subunits Ku 70/80, can specifically interact with p53R2 (Fig. S8). This result suggested that p53R2 may also be involved in ATR-mediated DNA damage repair.

Defects in DNA damage repair may cause genomic instability and predispose cells to cancer. Epidemiological studies have linked ATM mutations to a high risk of breast cancer (22). Moreover, a number of polymorphisms in the gene encoding p53R2 have been identified in esophageal squamous cell carcinoma and in colon carcinoma cells (23–25), but none of these mutations are associated with altered p53R2 activity. A possible identification of Ser12 mutation in breast cancer will further support the role of p53R2 in maintaining genomic integrity against cancer development. ATM and other proteins in its pathways have been studied as potential therapeutic targets to increase the sensitivity of cancer cells to DNA-damage agents (26). The development of p53R2 small molecular inhibitors to block Ser12 phosphorylation could be a potential therapy against malignancy.

Materials and Methods

Cells and UV Radiation. Cells were grown in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin G, and 100 μg/ml streptomycin. UV irradiation was performed with an UVC lamp (GBTS; General Electric). Dose rates were measured with a UVX Radiometer (UVP). For UV irradiation as well as for mock treatment, the growth medium was aspirated and the cell layer covered with a small amount of PBS solution. After treatment was completed, the growth medium was replenished.

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Supporting Information

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SI Materials and Methods

Real-Time Cell Proliferation Monitoring System. The principle of the ACEA RT-CES technology has been described previously (1). One thousand cells were seeded in each well of 16-well E-plate in 100 ml of culture media. Cell index (CI) is a quantitative measure of the number of cells attached to the sensors in the E plate. Cells were irradiated with UV (20 J/m²) when CI reaches a range of 1 to 1.2 at approximately 18 h after plating. CI was measured every 30 min for a period of 72 h.

Real-Time Quantitative PCR. Total DNA was extracted from the cells by using a DNeasy blood and tissue kit (Qiagen). mtDNA content was measured by quantification of a gene sequence encoding cytochrome c oxidase subunit I by real-time quantitative PCR using RNase-P nuclear gene for normalization. Real-time quantitative PCR was carried out using an ABI Prism 7000 sequence detection system. Primers specific to a region of the human cytochrome c oxidase subunit I or RNase-P gene were designed using ABI Primer Express software. Each PCR was performed in triplicate with the following profile: One cycle at 95°C for 10 min, and then 40 cycles at 95°C for 15 s and 60°C for 1 min (i.e., two-step protocol).

RNA Interference. The ATM siRNA (human) is commercially available from Santa Cruz Biotechnology. Briefly, 2 × 10⁶ cells were seeded per well in six-well culture plates filled with 2 ml antibiotic-free normal growth medium supplemented with FBS, then incubated at 37°C in a CO₂ incubator for 24 h. Cells were transfected with 7.2 µl of 10 µM ATM siRNA using a transfection reagent. Cells were incubated in the transfection medium for 5 h and then replaced with normal cell culture medium. The inhibition of ATM was measured by qPCR and Western blot.

dNTPs Pool Assays. An optimized dNTP pool assay has been described previously (1). Approximately 1 × 10⁶ cell pellets were harvested and added to 100 µl of 15% trichloroacetic acid. Supernatants were saved and extracted twice with 50 µl of 1,1,2-trichlorotrifluoroethane/trioctylamine (55:45). The reaction mixture (50 µl) of the assay contained 50 mM of Tris-HCl (pH 7.5), 10 mM MgCl₂, 5 mM DTT, 0.25 µM [³H]-dATP (for dCTP, dGTP, and dTTP pool detection) or [³H]-dTTP (for dATP pool detection), 0.2 units of Sequenase, and a diluted sample. After 20 min incubation, 40 µl aliquots were applied to circular Whatman DE81 ion exchange papers. After three washes, samples were counted in a liquid scintillation counter and compared with a standard sample prepared in the presence of 0, 0.25, 0.50, 0.75, and 1.0 pmol/ul each of dATP, dTTP, dGTP, and dCTP.


Fig. S1. p53R2 is not phosphorylated by JNK kinase after UV irradiation. Recombinant protein p53R2 (Top) and GST-cJun (1–77 aa; Bottom) as substrates were subjected to in vitro JNK immunocomplex kinase assay using the JNK immunoprecipitated from the same protein lysates as described in Fig. 1A. c-Jun is a known substrate for JNK (2).
Fig. S2. (A) Proteasome activity is required for UV-induced p53R2 degradation in cells defective in ATM. pEB57 (ATM-deficient) cells were incubated with or without the proteasome inhibitor MG-132 (50 μM) 30 min before UV treatment. At the indicated times, cell lysates were prepared and analyzed by immunoblotting with p53R2 antibody.
Fig. S3. Accumulation of p53R2 protein in MDM2-knockout and ATM-knockdown cells after UV exposure. p53⁻/⁻ or p53⁻/⁻:MDM2⁻/⁻ MEFs were transfected with ATM siRNA for 24 h. Cells were collected 2 h after irradiation with or without UV (20 J/m²). Cell lysates were collected and subjected to immunoblot analysis with antibodies against p53R2, MDM2, and GAPDH.
Fig. S4.  (A) The expression of transgenes (Myc-tagged WT-, S72A-, S112A-p53R2, or empty vector) in stable KB cell lines was analyzed by immunoblotting analysis using an anti-Myc antibody. (B) Dynamic monitoring of cellular responses to UV. Cells (1 × 10⁶) stably expressing WT-, S72A-p53R2, or empty vector were seeded into 16-well E-plates. Cell growth was monitored by the RT-CES system (ACEA Biosciences) and indicated as CI. (C) Real-time PCR quantification of the mtDNA in cells stably expressing empty vector, WT-, or S72A-p53R2 at the indicated times after UV (20 J/m²) exposure. The measurements were repeated three times. Error bars indicate SD.
Fig. S5. Interaction between p53, p53R2, and ATM and increased interaction between p53 and p53R2. (A) Co-IP of endogenous ATM and p53 by antibodies to p53R2 and enhanced interaction between endogenous ATM, p53, and p53R2 after UV irradiation (20 J/m²). The same experiment was performed in Fig. 3 except that p53R2 antibody was used for co-IP and anti-ATM, anti-p53, and anti-p53R2 antibodies were used to immunoblot. (B) Enhanced interaction between exogenous p53 and S72A-p53R2 mutant. The same experiment performed in Fig. 2 except that p53-GFP instead of MDM2 expression plasmid is co-transfected with other plasmids into 293 cells, and anti-ATM and anti-p53 antibodies were used to immunoblot the Myc-tagged immunoprecipitates.
Fig. S6. Overexpressed S72A-p53R2 reduced dNTP pools in cells. Analysis of dNTP pools after UV irradiation (20 J/m²). Cells were harvested and analyzed for intracellular dNTP concentration, including dATP, dCTP, dGTP, and dTTP. The level of dNTPs was adjusted by cell number.
Fig. S7. p53R2 interacts with the MRE11-RAD50-NBS1 complex but not BRCA1. Co-IP of endogenous MRE11 complex by anti-p53R2 antibody. Cell lysates were isolated from KB cells 30 min before and after doxycycline (Dox; 20 µM) treatment, immunoprecipitated by anti-p53R2 antibody, and immunoblotted with anti-MRE11, anti-Rad50, anti-NBS1, anti-BRCA1, or anti-p53R2 antibodies.
p53R2 specifically interacts with the ATR regulatory partner ATR-interacting protein (ATRIP) but not with DNA-PK regulatory subunits Ku 70 and Ku 80. Co-IP of endogenous MRE11 complex by anti-p53R2 antibody. Cell lysates were isolated from KB cells 30 min before and after doxycycline (Dox; 20 μM) treatment, immunoprecipitated by anti-p53R2 antibody, and immunoblotted with anti-ATRIP, anti-Ku 70/80, or anti-p53R2 antibodies.