Def1 Promotes the Degradation of Pol3 for Polymerase Exchange to Occur During DNA-Damage–Induced Mutagenesis in *Saccharomyces cerevisiae*

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**Abstract**

DNA damages hinder the advance of replication forks because of the inability of the replicative polymerases to synthesize across most DNA lesions. Because stalled replication forks are prone to undergo DNA breakage and recombination that can lead to chromosomal rearrangements and cell death, cells possess different mechanisms to ensure the continuity of replication on damaged templates. Specialized, translesion synthesis (TLS) polymerases can take over synthesis at DNA damage sites. TLS polymerases synthesize DNA with a high error rate and are responsible for damage-induced mutagenesis, so their activity must be strictly regulated. However, the mechanism that allows their replacement of the replicative polymerase is unknown. Here, using protein complex purification and yeast genetic tools, we identify Def1 as a key factor for damage-induced mutagenesis in yeast. In *in vivo* experiments we demonstrate that upon DNA damage, Def1 promotes the ubiquitylation and subsequent proteasomal degradation of Pol3, the catalytic subunit of the replicative polymerase δ, whereas Pol31 and Pol32, the other two subunits of polymerase δ, are not affected. We also show that purified Pol31 and Pol32 can form a complex with the TLS polymerase Rev1. Our results imply that TLS polymerases carry out DNA lesion bypass only after the Def1-assisted removal of Pol3 from the stalled replication fork.

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Abbreviations: GST, glutathione S-transferase; HA, hemagglutinin; MMS, methyl methanesulfonate; PCNA, proliferating cell nuclear antigen; Pol, polymerase; TAP, tandem affinity purification; TLS, translesion synthesis; UV, ultraviolet light.

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**Introduction**

The stalling of the replication machinery that occurs as a consequence of encountering unrepaired DNA damages is a challenging problem for cells. Stalled replication forks can undergo DNA breakage and recombination that can lead to chromosomal rearrangements and cell death. To ensure survival, cells have evolved different mechanisms that can sustain DNA replication on damaged templates. These so-called DNA damage tolerance or TLS polymerases synthesize DNA with a high error rate and are responsible for damage-induced mutagenesis, at its lysine-164 residue at the stalled replication fork [7]. Monoubiquitylated PCNA activates the Rev3, and the Rad30-dependent subpathways involving TLS polymerases, whereas further polyubiquitylation of PCNA on the same residue through a lysine-63–linked chain by the Rad5–Mms2–Ubc13 ubiquitin–conjugase–ligase complex activates the Rad53 subpathway [7,8]. Genetic experiments suggest that the Rad5 branch operates through template switching, where the newly synthesized strand of the undamaged sister duplex serves as a template to bypass the lesion [9]. Rad5, a SWI–SNF family member helicase, most probably directly promotes this process through its fork-reversal activity [10]. The *RAD30*-encoded DNA polymerase η (Polη) is unique in its ability to efficiently and accurately synthesize through UV-induced cyclobutane pyrimidine dimers [11]. In accordance with its role in the error-free bypass of UV lesions, a defect of Polη in yeast confers an increase in UV-induced mutations, and in humans it causes the cancer-prone syndrome, the variant form of xeroderma pigmentosum [12–14]. Besides UV-lesions, Polη can...
bypass several DNA distorting lesions with varying accuracy [2]. The mutagenic branch involves Rev1 and Rev7, besides Rev3, and the lack of either protein causes immutability [15]. The Rev1 protein is a DNA polymerase with limited ability to insert C residues [16]. Its catalytic activity is dispensable for most induced mutagenesis events, suggesting a mainly structural role for Rev1 [17]. Rev3 together with Rev7 forms DNA polymerase \( \text{f} \) (Pol\( _f \)) [18]. Rev7 is an accessory protein, whereas Rev3 is the catalytic subunit. Pol\( _f \) has the ability to efficiently extend from mispaired nucleotides and from nucleotides inserted opposite different DNA lesions [2].

TLS polymerases synthesize DNA with a high error rate and are responsible for introducing mutations into the genome during DNA damage bypass, so their replacement of the replicative polymerase must be tightly regulated. However, our understanding of the polymerase switch at DNA damage sites is elusive. Pol\( _g \), Rev1, and Pol\( _f \) were shown to interact with PCNA, and it was suggested that through these interactions TLS polymerases could get access to the replication fork [19–21]. Also, the interaction with PCNA was shown to be essential for the in vivo function of all three polymerases. Though PCNA binding can give access to TLS polymerases to the replication fork, the mechanism that allows them to actually take over DNA synthesis from the replicative polymerase during DNA lesion bypass is still unknown.

In this study, we identify Def1 as an indispensable regulator of induced mutagenesis. We show that Def1 promotes the ubiquitylation and subsequent proteasomal degradation of the catalytic subunit of the replicative polymerase after DNA damage treatment. We demonstrate that the noncatalytic subunits of the replicative polymerase are not affected by UV-induced degradation and that they can form a complex with the TLS polymerase.

**Results**

**Rad5 Forms a Stable Complex with Def1 upon MMS Treatment**

In searching for new factors affecting DNA damage tolerance, we aimed to identify new interacting partners of Rad5. Therefore, we performed tandem affinity purification (TAP) of Rad5 together with its complexes. For that purpose, we introduced a TAP tag, consisting of a calmoduline binding peptide and two IgG binding domains of protein A separated by a TEV protease cleavage site, at the C-terminus of Rad5 at the chromosomal locus. We purified Rev1. Based on our results we propose a new model for polymerase exchange at stalled replication forks.
Rad5 and its interacting partners through the two affinity tags under native conditions. To facilitate the formation of damage bypass complexes, we applied 0.02% methyl methanesulfonate (MMS) for 2 h before collecting the cells. Surprisingly, without treatment only one prominent, specific band was visible in the final, highly purified fraction on the Coomassie-stained gel (Figure 1), which was identified by mass spectrometry as the tagged Rad5 itself. However, after MMS treatment two prominent bands appeared on the gel; the lower mobility band was again Rad5, whereas the higher mobility band was identified as Def1. Repetition of the experiment yielded the same result that Def1 copurified with Rad5, but only after treating the cells with MMS. It suggested that either DNA-damage–induced posttranslational modification of Rad5 and/or Def1 or a third, damage-specific factor was necessary to promote the formation of the complex.

DEF1 Functions in the RAD6-Mediated DNA Damage Tolerance

Rad5 mediates an error-free DNA damage tolerance pathway under the control of Rad6–Rad18, whereas Def1 has a role in promoting the proteolytic degradation of stalled RNA polymerase.

Figure 2. DEF1 participates in the REV3 branch of the RAD6-governed DNA damage tolerance. (A–E) Epistatic analysis of DEF1 with mutants of the different branches of the RAD6 pathway upon UV irradiation. Standard deviations are indicated. (F–J) Epistatic analysis of the same mutants upon MMS treatment. (K, L) Genetic interactions of RAD30 with MMS2 and REV3 upon MMS treatment. All experiments were repeated at least three times.

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II and in telomere maintenance [22,23]. To further establish a
connection between Rad5 and Def1, we analyzed the genetic
relations between
DEF1
and
RAD5
, and members of all three
branches of the
RAD6
-governed pathway upon DNA damage.

After treating the cells with UV, the sensitivity of the
def1 rad6
double deletion strain did not exceed that of the
rad6
mutant,
pointing to an epistatic relationship between
DEF1
and
RAD6
(Figure 2A). The higher resistance seen with the double mutant
might originate from other functions of these multitask proteins, as
the
def1 rad18
strain showed the same sensitivity as
rad18
(unpublished data), fortifying the involvement of
DEF1
in the
RAD6-dependent DNA damage tolerance. Surprisingly, the
def1 rad5
double deletion strain displayed much higher sensitivity than
any of the corresponding single mutants, indicating that
DEF1
acted outside of the
RAD5-dependent subpathway (Figures 2B). The higher resistance seen with the double mutant
might originate from other functions of these multitask proteins, as
def1 rad5
strain showed the same sensitivity as
rad5
(unpublished data), fortifying the involvement of
DEF1
in the
RAD5-dependent DNA damage tolerance. Surprisingly, the
def1 rad5
double deletion strain displayed much higher sensitivity than
any of the corresponding single mutants, indicating that
DEF1
acted outside of the
RAD5-dependent subpathway (Figures 2B). This was verified by the hypersensitivity of the
def1 mms2
strain over the single mutants (Figure 2C). Also, the
def1 rad30
double mutant was more sensitive to UV than either
def1
or
rad30,
implying that
DEF1
functioned independently of
RAD30
(Figure 2D). Nevertheless, the
def1 rev3
strain exhibited the same sensitivity as the
rev3
strain, which indicated an epistatic relationship between
DEF1
and
REV3
(Figure 2E). We carried out similar experiments using MMS instead of UV as a DNA
damage source (Figures 2F–J). Upon MMS treatment
DEF1
showed epistasis with
RAD6
and
REV3,
but its deletion further sensitised
rad5
and
mms2,
proving again the involvement of
DEF1
in the mutagenic branch of the
RAD6-governed DNA damage tolerance. However,
DEF1
also showed epistasis with
RAD30,
as
the double mutant was as sensitive as the
def1
single mutant. We note that this reflected a real epistatic relationship, as although
rad30
itself was not sensitive to MMS, only at very high doses, it
was hypersensitive with
mms2,
but also showed epistasis with
REV3
(Figure 2K,1). That means that in the bypass of MMS-induced DNA lesions,
RAD30
works together with the members of the
REV3
branch. In conclusion, our data strongly suggested that
DEF1
participated in the
REV3-dependent mutagenic branch of the
RAD6–RAD18-regulated DNA damage tolerance.

DNA-Damage–Induced Mutagenesis Is Abolished in
def1
Deletion Mutants

The TLS polymerases of the
REV3
branch are responsible for
virtually all damage-induced mutagenesis; consequently, inactiva-
tion of either one causes a strong antimutator effect [15]. To prove
that
DEF1
belonged to the
REV3
branch, we measured the rate of
UV-induced mutations in
def1
strains. In keeping with the results of
the epistasis analysis, induced mutagenesis was completely abolished
in
def1
(Figure 3). In fact,
def1
was even more defective than the
rev3
strain. Additional deletion of
DEF1
in
mms2
also eliminated induced mutagenesis, though
mms2
by itself causes high mutagenesis, most
probably because in the absence of the error-free branch, lesions are
channelled to the
REV3-dependent mutagenic pathway. Ectopic
expression of Def1 in
def1
cells restored close to wild-type–level mutagenesis, confirming that the immutability was in fact due to the
absence of
DEF1.
We obtained the same results using MMS instead of UV (unpublished data). From these we concluded that
DEF1
played an essential role in induced mutagenesis.
Pol3 Is Degraded upon DNA Damage by a Def1-Dependent Manner

For the REV3 branch to operate, the TLS polymerases of the branch have to take over synthesis from the replicative polymerase stalled at a DNA lesion site, a central but poorly understood step in DNA lesion bypass. Because Def1, unlike other members of the REV3 branch, is not a DNA polymerase, we surmised that it might facilitate the exchange between the TLS and the replicative polymerases. As Def1 played a role in the ubiquitylation of stalled RNA polymerase II [22], we considered the possibility that, similarly, it could mediate ubiquitylation of the stalled replicative DNA polymerase. Ubiquitylation then could lead to polymerase switch by either playing a regulatory role as in the case of DNA-damage–induced ubiquitylation of PCNA [7], or it could result in protein removal through degradation. To test these possibilities, we followed the fate of the replicative polymerase during DNA damage bypass by monitoring Pol3, the catalytic subunit of the replicative DNA polymerase δ (Polδ) during cell cycle in UV-treated, synchronized yeast cultures. Importantly, we observed a transient decrease in the level of Pol3 upon UV irradiation as opposed to normal growth conditions, and the degree of degradation correlated with the applied UV doses (Figure 4).

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Def1 Induces the Ubiquitylation and Proteasomal Degradation of Pol3

The most plausible explanation for the transient decrease of Pol3 would be that Pol3 underwent regulated protein degradation induced by UV. The majority of regulated proteolysis takes place in the proteasome in eukaryotic cells. To resolve whether the decrease in the Pol3 protein level was due to protein degradation mediated by the proteasome, we supplemented the growth media with the proteasome inhibitor MG132. Indeed, in the presence of MG132, the UV-induced degradation of Pol3 could not be observed (Figure 5D). To add further evidence, we applied a temperature-sensitive rpn7 mutant displaying defects in proteasome function at high temperature (37°C) but behaving like wild-type at low temperature (25°C) [24]. Using this mutant we could not detect degradation at the restrictive high temperature contrary to the permissive low temperature (Figure 6A), whereas in the RP7 strain degradation occurred at both temperatures (unpublished data). These results demonstrated that the proteasome was responsible for the UV-induced degradation of Pol3.

Ubiquitylation is a major signal for proteasomal protein degradation. To show ubiquitylation of Pol3, N-terminally 7 histidine-tagged ubiquitin was expressed in yeast cells and ubiquitylated proteins from cell extracts prepared after irradiating cells with UV were enriched on nickel beads. Indeed, we could...
detect polyubiquitylated forms of Pol3 upon UV irradiation in wild-type cells, but not in def1 and rad6 cells (Figure 7B and unpublished data).

Pol31 and Pol32 Are Not Subject to UV-Induced Degradation

Pol\(\delta\) is a heterotrimer and consists of two noncatalytic subunits, Pol31 and Pol32, besides Pol3 [25]. Pol31, like Pol3, is essential for cell viability, but Pol32 is a nonessential subunit. Pol3 forms a stable complex with Pol31, and Pol32 is attached to this complex through its interaction with Pol31 [26]. We aimed to examine whether the whole Pol\(\delta\) enzyme was subject to UV-induced proteolysis, or it affected only the catalytic subunit. We found that contrary to Pol3, Pol31 and Pol32 were not affected by UV-induced degradation (Figure 5F and 5G).

### Figure 5. Pol3 degradation depends on RAD6 and DEF1.

Cultures were synchronized by α-factor, UV-irradiated with 150 J/m\(^2\), and released back to growth media. Proteins from whole cell extracts, prepared from 1 ml of cell culture collected at the indicated time points after UV treatment, were analyzed by Western blotting. Anti-HA antibody was used to detect HA-tagged Pol3 (A to E), Pol31 (F), or Pol32 (G). Cell cycle progression was monitored by Clb2 cyclin levels, and PGK served as a loading control. The level of Pol3 relative to PGK is shown at the bottom of each panel. doi:10.1371/journal.pbio.1001771.g005

Pol31 and Pol32 Can Form a Complex with Rev1

Taken together, these results suggested that during DNA damage bypass, Pol31 and Pol32 remained at the stalled fork. We postulated that a TLS polymerase could take the place of Pol3.
and carry out lesion bypass in complex with Pol31 and Pol32. To test this idea, we examined whether Pol31 and Pol32 together could form a complex with Rev1 in in vitro assays using purified proteins. We chose Rev1, because it had been suggested to function as a scaffold in TLS, based on its interaction in yeast with Pol\(_{g}\) and Pol\(_{f}\) [27,28], and in mouse and human cells with Pol\(_{g}\), Poli, and Polk [29,30]. Also, it has already been shown to interact with Pol32 [31]. In GST pull-down assays we added Pol31 and Rev1 to GST–Pol32 immobilised on glutathione–Sepharose affinity beads, and after incubation bound proteins were released from the beads by glutathione. As shown in Figure 7B lanes 1–4, both Pol31 and Rev1 eluted together with GST–Pol32, indicating that these proteins formed a complex together. In control experiments using GST instead of GST–Pol32, only GST was present in the elution fraction, confirming that the interaction between Pol31, Pol32, and Rev1 was specific (Figure 7B, lanes 5–8). In conclusion, purified Pol31, Pol32, and Rev1 could interact directly and form a stable multisubunit protein complex.

**Discussion**

In this study we identified a DNA-damage–induced complex of Rad5 with Def1. Our genetic studies placed *DEF1* in the *RAD6–RAD18*-dependent DNA damage tolerance pathway, where it played an indispensable role during induced mutagenesis. We established that Pol3, the catalytic subunit of the replicative DNA polymerase Pol\(_{d}\), was degraded upon UV irradiation. We presented evidence that degradation of Pol3 was the result of polyubiquitylation-mediated proteasomal degradation, and it was dependent on *DEF1* under the higher control of *RAD6*. Conversely, Pol31 and Pol32, the other two subunits of Pol\(_{d}\), were not degraded. We also demonstrated that Pol31 and Pol32 together could form a stable complex with the TLS polymerase Rev1. Based on these results, we propose a new model for polymerase exchange at stalled replication forks (Figure 8). During replication, when Pol\(_{d}\) stalls at a DNA lesion, PCNA gets ubiquitylated by Rad6–Rad18. Monoubiquitylated PCNA acti-
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Pol3 is a DNA polymerase that is mainly specialized for the error-free bypass of UV lesions, so it is reasonable to assume that Pol3 should have preference over the slower kinetic damage bypass to occur, Pol3 has to be removed so that the TLS polymerases could form a stable complex with Pol31 and Pol32. This would also explain the epistasis of RAD30 with Def1 in the bypass of MMS-induced DNA lesions, as the efficiency of incorporation by Polη is reduced ~20-fold opposite 6-O-methylguanine and ~1,000-fold opposite an abasic site [40,41].

We detected a very stable DNA-damage-induced complex formation of Rad5 with Def1. However, our genetic data placed the two genes into two alternative DNA damage tolerance pathways, both governed by Rad6. We hypothesize that the Def1–Rad5 complex might coordinate the activity of the two subpathways in response to DNA damage. A similar role of Def1 during transcription was suggested, where Def1 assisted in the degradation of the RNA polymerase stalled at DNA damage sites, and probably coordinated the repair mechanisms through its interaction with Rad26 [22].

The high conservation between elements of DNA lesion bypass from yeasts to humans, including the Rad6–Rad18 and Rad5–Mms2–Ubc13 complexes and their enzymatic activities, the TLS polymerases, and PCNA ubiquitylation [42], suggests that DNA-damage–induced selective degradation of the catalytic subunit of the replicative DNA polymerase drives polymerase exchange in higher eukaryotes as well. The role of TLS polymerases in mutagenesis and in cancer makes it highly important to uncover further details of polymerase exchange, to identify and investigate further factors that affect Pol3 degradation, and to check the existence of a similar mechanism in human cells.

Materials and Methods

Yeast Strains and Plasmids

The wild-type strain (BY4741) and its single deletion derivatives for the genetic studies were obtained from the Euroscarf collection. Chromosomally C-terminally tagged POL3, POL31, and POL32 with three copies of the hemagglutinin epitope tag (3-HA) were
created by a PCR-based strategy [43] in EMY74.7 (MATa, his3-D1, leu2-3, -112, trp1-D, ura3-52) strain, made bar1Δ. Additional deletions were generated by gene replacement. RAD5 was TAP tagged in BJ5464 by the same PCR-based strategy using pBS1539 [44]. BJ5464 was also used for protein overexpression. The rpn7-3 mutant and its corresponding W303 wild-type strain [24] were used in experiments showing the effect of temperature-sensitive inhibition of the proteasome. Polyubiquitylation of Pol3 was shown in MHY500 strain background [45]. For complementation in yeast, Def1 was expressed from the centromeric vector pID394 (p416ADH backbone [46]). For protein purification, Pol31, Pol32, and Rev1 were overexpressed in N-terminal GST fusion from pID370, pID458, and pID460, respectively (pBJ842 backbone [47]). In the plasmid pRS426-pCUP1–His7–Ubiquitin (G76A) [48], the mutation was reversed by site-directed mutagenesis, resulting in plasmid pID198.

Sensitivity Assays

For qualitative analysis of sensitivity to MMS, cells were serial diluted and spotted onto YPD plates containing defined amounts of MMS and grown at 30°C for 3–5 d. For quantification, cells were spread onto YPD plates at appropriate dilutions and irradiated with UV light (254 nm) for varying times to apply the specified dosage. Plates were incubated in the dark at 30°C, and colonies were counted after 3–5 d.

UV-Induced Mutation Rate

UV-induced forward mutation frequencies at the CAN1 locus were measured by comparing the numbers of can1Δ colonies at given UV doses, selected on synthetic complete medium without arginine and containing canavanine, with the numbers of survivors on complete synthetic medium, exposed to the same UV doses.
Cell Synchronisation
Logarithmically growing cells in YPD at 30°C were arrested at *A*$_{600}$:0.8 in G1 by 100 ng/μl α-factor (Sigma) for 2–4 h, washed, resuspended in phosphate buffered saline, and divided into Petri dishes for UV irradiation. Half of the cultures were irradiated with the given UV dose, and the other half served as untreated control. Cells were released back into growth medium containing 50 μg/ml pronase (Calbiochem) to inactivate any residual α-factor. For experiments showing polyubiquitylation of Pol3, the growth media always contained 100 μM CuSO$_4$ to induce 7His-ubiquitin expression. Samples were taken at given time points after UV treatment for whole cell extract preparation. Experiments involving MG132 (Sigma) were done in *Δpdr5* background. MG132 (50 μM) was added to the α-factor synchronized cultures 1 h before UV irradiation. The *Δpdr5-α* mutant and its isogenic wild-type strain were grown at 25°C. Half of the culture was kept at 25°C by 100 ng/ml α-factor, and the other half was shifted to 37°C. At *A*$_{600}$:0.8 cultures were synchronised by α-factor for 3 h and processed as detailed above.

Protein Techniques and Antibodies
Whole cell extracts were prepared according to a trichloroacetic acid (TCA) protein precipitation method [43] except that after TCA precipitation, pellets were washed with ice-cold acetone, air-dried, and resuspended in 1× Laemmli sample buffer before loading to an 8% poly-acrylamide gel. Polyubiquitylated Pol3 was detected using denaturing NiNTA chromatography as described in [49]. Antibodies against HA (Gene Tex), Cbi2 (Santa Cruz), PGK (Molecular Probes), and ubiquitin (Santa Cruz) were used. Pol31, Pol32, and Rev1 were overexpressed in N-terminal fusion with GST and purified on glutathione–Sepharose 4B beads following the protocol in [45], with the exception that in the case of Rev1, 0.1% Triton X-100 was added to the lysis after breaking the cells. In the case of Pol31 and Rev1, the GST tag was removed by PreScission protease cleavage in the elution step of purification. For complex formation, GST–Pol32 (3 μg) immobilized on glutathione–Sepharose beads was incubated with purified Pol31 (5 μg) and Rev1 (3 μg), overnight on ice in buffer containing 50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 10% glycerol, 0.01% Nonidet P-40. Beads were washed five times with the same buffer, and bound proteins were eluted in the same buffer containing 20 mM reduced glutathione. Various fractions were analyzed by SDS/PAGE.

TAP
Four liters of yeast culture were grown to logarithmic phase in synthetic complete medium, and at *A*$_{600}$:0.8, half of the culture was treated with 0.02% MMS for 2 h before harvesting. TAP purification was carried out as described [44] with the following modifications: cells were broken in 1× YBB (50 mM Tris/HCl pH 7.3, 50 mM KCl, 100 mM NaCl, 10% sucrose) supplemented with protease inhibitors. After clarifying the lysate with ultracentrifugation for 1 h with 100,000 g, 2-mercaptoethanol was added to 8.5 mM, Nonidet P-40 to 0.01%, and NaCl to 500 mM final concentration, and the lysate was transferred into an IgG Sepharose bead (Amersham)-filled column. In later steps the protocol was followed. Briefly, bound fraction was eluted with TEV protease cleavage. The elution fraction was applied on calmodulin beads, and bound proteins were recovered by eluting in EGTA-containing buffer. Proteins were concentrated and analysed on a 6%–12% gradient sodium dodecyl sulphate polyacrylamide gel stained with Coomassie blue R-250. Excised protein bands were identified by MALDI-TOF mass spectrometry after trypsin digestion. Eleven peptides of the higher mobility band matched yeast Def1 (55% coverage) and they covered 18% of the Def1 sequence.

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Author Contributions
The author(s) have made the following declarations about their contributions: Conceived and designed the experiments: LH IU. Performed the experiments: AD VKG MH LH IU. Analyzed the data: AD VKG MH LH IU. Wrote the paper: IU.

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