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Role for a bidentate ribonuclease in the initiation step of RNA interference

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RNA interference (RNAi) is the mechanism through which double-stranded RNAs silence cognate genes[1–5]. In plants, this can occur at both the transcriptional and the post-transcriptional levels[1,2,5]; however, in animals, only post-transcriptional RNAi has been reported to date. In both plants and animals, RNAi is characterized by the presence of RNAs of about 22 nucleotides in length that are homologous to the gene that is being suppressed[6–8]. These 22-nucleotide sequences serve as guide sequences that instruct a multicomponent nuclease, RISC, to destroy specific messenger RNAs[6]. Here we identify an enzyme, Dicer, which can produce putative guide RNAs. Dicer is a member of the RNase III family of nucleases that specifically cleave double-stranded RNAs, and is evolutionarily conserved in worms, flies, plants, fungi and mammals. The enzyme has a distinctive structure, which includes a helicase domain and dual RNase III motifs. Dicer also contains a region of homology to the RDE1/QDE2/ARGONAUTE family that has been genetically linked to RNAi[9,10].

Biochemical studies have suggested that post-transcriptional gene silencing (PTGS) is accomplished by a multicomponent nuclease that targets mRNAs for degradation[6,8,11]. The specificity of this complex may derive from the incorporation of a small guide sequence that is homologous to the mRNA substrate[6]. These ~22-nucleotide RNAs, originally identified in plants that were actively silencing transgenes[7], have been produced during RNAi *in vitro* using an extract prepared from *Drosophila* embryos[8]. Putative guide RNAs can also be produced in extracts from *Drosophila* S2 cells (Fig. 1a). To investigate the mechanism of PTGS, we have performed both biochemical fractionation and candidate gene approaches to identify the enzymes that execute each step of RNAi.

Our previous studies resulted in the partial purification of an enzyme complex, RISC, which is an effector nuclease for RNA interference[6]. This enzyme was isolated from *Drosophila* S2 cells in which RNAi had been initiated *in vivo* by transfection with double-stranded RNA (dsRNA). We first investigated whether the RISC enzyme, and the enzyme that initiates RNAi through processing of dsRNA into 22-nucleotide sequences, are distinct activities. RISC activity could be largely cleared from extracts by high-speed centrifugation (100,000*g* for 60 min), whereas the activity that produces 22-nucleotide sequences remained in the supernatant (Fig. 1b, c). This simple fractionation indicates that RISC and the 22-nucleotide sequence-generating activity may be separable. However, it seems probable that these enzymes interact at some point during the silencing process, and it remains possible that initiator and effector enzymes share common subunits.

RNase III family members are among the few nucleases that show specificity for dsRNA[12]. Analysis of the *Drosophila* and *Caenorhabditis elegans* genomes reveals several types of RNase III enzymes. First is the canonical RNase III, which contains a single RNase III signature motif and a dsRNA-binding domain (dsRBD; for example RNC_CAEEL). Second is a class represented by Drosha[13], a *Drosophila* enzyme that contains two RNase III motifs and a dsRBD (CeDrosha in *C. elegans*). A third class contains two RNase III signatures and an amino-terminal helicase domain (for example, *Drosophila* CG4792 and CG6493; *C. elegans* K12H4.8), which had been proposed as potential RNAi nucleases[14,20]. We tested representatives of all three classes for the ability to produce discrete RNAs of ~22 nucleotides from dsRNA substrates.

To test the dual RNase III enzymes, we prepared variants of Drosha and CG4792 tagged with the T7 epitope. These were expressed in transfected S2 cells and isolated by immunoprecipitation using antibody–agarose conjugates. Treatment of the dsRNA with the CG4792 immunoprecipitate yielded fragments of about 22 nucleotides, similar to those produced in either the S2 or embryo extracts (Fig. 2a). Neither the activity in extract nor that in immunoprecipitates depended on the sequence of the RNA substrate, as dsRNAs derived from several genes were processed equivalently (see Supplementary Information). Negative results

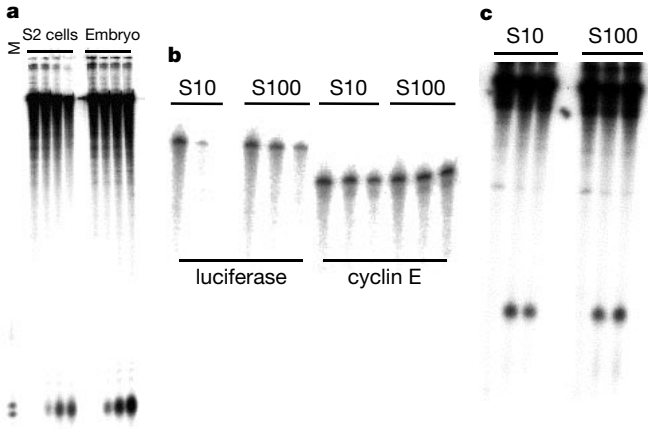

Figure 1 Generation of 22-nucleotide sequences and degradation of mRNA by distinct enzymatic complexes. **a**, Extracts prepared from for 0–12 h *Drosophila* embryos or *Drosophila* S2 cells. Extracts were incubated for 0, 15, 30 or 60 min (left to right) with a uniformly labelled dsRNA. A 22-nucleotide marker prepared by *in vitro* transcription of a synthetic template is indicated (M). **b**, Whole-cell extracts from S2 cells transfected with luciferase dsRNA. S10 represents our standard RISC extract[6]. S100 extracts were prepared by additional centrifugation of S10 extracts for 60 min at 100,000*g*. Assays for mRNA degradation[6] were performed for 0, 30 or 60 min (left to right in each set) with either a single-stranded luciferase mRNA or a single-stranded cyclin E mRNA, as indicated. **c**, S10 or S100 extracts incubated with cyclin E dsRNAs for 0, 60 or 120 min (left to right).

were obtained with Drosha and with immunoprecipitates of a DExH box helicase (Homeless[15]; see Fig. 2a and b). Western blotting confirmed that each of the tagged proteins was expressed and immunoprecipitated similarly (see Supplementary Information). Thus, we conclude that CG4792 may carry out the initiation step of RNAi by producing guide sequences of about 22 nucleotides from dsRNAs. Because of its ability to digest dsRNA into uniformly sized, small RNAs, we have named this enzyme Dicer (*Dcr*). *Dicer* mRNA is expressed in embryos, in S2 cells and in adult flies, which is consistent with the presence of functional RNAi machinery in all of these contexts (see Supplementary Information).

An antiserum directed against the carboxy terminus of the Dicer protein (Dicer-1, CG4792) could immunoprecipitate a nuclease activity from either the *Drosophila* embryo extracts or from S2 cell lysates that produced RNAs of about 22 nucleotides from dsRNA substrates (Fig. 2c). The putative guide RNAs that are produced by the Dicer-1 enzyme precisely co-migrate with 22-nucleotide sequences that are produced in extract, and with 22-nucleotide sequences that are associated with the RISC enzyme (Fig. 2d, f). The enzyme that produces guide RNAs in *Drosophila* embryo extracts is ATP dependent[8]. Depletion of this cofactor resulted in a roughly sixfold reduction of dsRNA cleavage rate and in the production of RNAs with a slightly lower mobility. Of note, both Dicer-1 immunoprecipitates and extracts from S2 cells require ATP for the production of ~22-nucleotide sequences (Fig. 2d). We did not observe the accumulation of lower-mobility products in these cases, although we did routinely observe these in ATP-depleted embryo extracts. The requirement of this nuclease for ATP is an unusual property, and may indicate that unwinding of guide RNAs by the helicase domain is required for the enzyme to act catalytically.

For efficient induction of RNAi in *C. elegans* and in *Drosophila*, the initiating RNA must be double-stranded and must also be several hundred nucleotides in length[4]. Similarly, Dicer was inactive against single-stranded RNAs regardless of length (see Supplementary Information). The enzyme could digest both 200- and 500-nucleotide dsRNAs, but was significantly less active with shorter substrates (see Supplementary Information). In contrast, *Escherichia coli* RNase III could digest to completion dsRNAs of 35 or 22 nucleotides (data not shown). This suggests that the substrate preferences of the Dicer enzyme may contribute to, but not wholly determine, the size dependence of RNAi.

To determine whether the Dicer enzyme is involved in RNAi *in vivo*, we depleted Dicer activity from S2 cells and tested the effect on dsRNA-induced gene silencing. Transfection of S2 cells with a

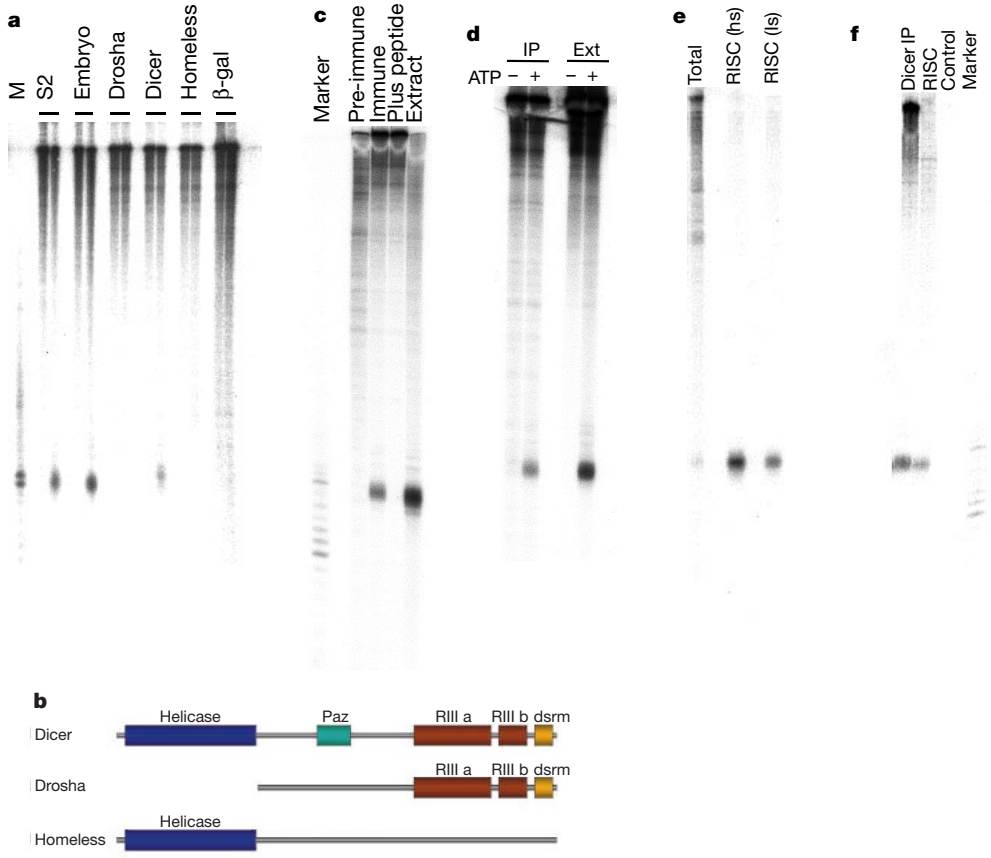

Figure 2 Production of 22-nucleotide sequences by CG4792/Dicer. **a**, *Drosophila* S2 cells transfected with plasmids that direct expression of T7-epitope-tagged versions of Drosha, CG4792/Dicer-1 and Homeless or untagged β-galactosidase. Proteins were immunoprecipitated and incubated with *cyclin E* dsRNA for 0 or 60 min. Reactions in *Drosophila* embryo and S2 cell extracts are shown. **b**, Domain structures of CG4792/Dicer-1, Drosha and Homeless. **c**, Immunoprecipitates prepared from detergent lysates of S2 cells using Dicer antiserum. As controls, similar preparations were made with a pre-immune serum and an immune serum that had been pre-incubated with an excess of antigenic peptide. Cleavage reactions in which each of these precipitates was incubated with a ~500 nucleotide fragment of *Drosophila* cyclin E are shown. An incubation of the substrate in *Drosophila* embryo extract is shown. **d**, Dicer immunoprecipitates incubated with dsRNA substrates in presence or absence of ATP. The same substrate was also incubated with ATP-added or ATP-depleted S2 extracts. **e**, *Drosophila* S2 cells transfected with uniformly, ^{32}P-labelled dsRNA corresponding to the first 500 nucleotides of GFP. RISC complex was affinity purified using a histidine-tagged version of *Drosophila* Ago-2, a component of the RISC complex (Hammond *et al.*, manuscript in preparation). RISC was isolated under ribosome-associated (ls, low salt) or soluble, ribosome-extracted (hs, high salt) conditions[6]. The spectrum of labelled RNAs in the total lysate is shown. **f**, Comparison of guide RNAs produced by incubation of dsRNA with a Dicer immunoprecipitate, with guide RNAs present in affinity-purified RISC complex. These co-migrate on a gel that has single-nucleotide resolution. The control lane shows an affinity selection for RISC from cells transfected with labelled dsRNA, but not with the epitope-tagged *Drosophila* Ago-2.

mixture of dsRNAs homologous to the two *Drosophila* Dicer genes (CG4792 and CG6493) resulted in a roughly 6–7-fold reduction of Dicer activity either in whole-cell lysates or in Dicer-1 immunoprecipitates (Fig. 3a and b). Transfection with a control dsRNA (murine caspase-9) had no effect. Qualitatively similar results were seen if Dicer mRNA was examined by northern blotting (data not shown). Depletion of Dicer substantially compromised the ability of cells to silence an exogenous, green fluorescent protein (GFP) transgene by RNAi (Fig. 3c). These results indicate that Dicer may be involved in RNAi *in vivo*. The lack of complete inhibition of silencing may result from an incomplete suppression of Dicer or may indicate that *in vivo* guide RNAs may be produced by more than one mechanism.

Our results indicate that the process of RNAi can be divided into at least two distinct steps. Initiation of PTGS would occur on processing of a dsRNA by Dicer into ~22-nucleotide guide sequences, although we cannot formally exclude the possibility that another Dicer-associated nuclease may participate in this process. These guide RNAs would be incorporated into a distinct nuclease complex (RISC) that targets single-stranded mRNAs for degradation. An implication of this model is that the guide sequences are themselves derived directly from the dsRNA that triggers the response. In accord with this model, we have shown that ^{32}P-labelled, exogenous dsRNAs that have been introduced into S2 cells by transfection are incorporated into the RISC enzyme as 22-nuclotide sequences (Fig. 2e).

A notable feature of the Dicer family is its evolutionary conservation. Homologues are found in *C. elegans* (K12H4.8), *Arabidopsis* (for example, CARPEL FACTORY[16], T25K16.4 and AC012328_1), mammals (Helicase-MOI[17]) and *Schizosaccharomyces pombe* (YC9A_SCHPO) (see Supplementary Information for comparisons). In fact, the human Dicer family member is capable of generating ~22-nucleotide RNAs from dsRNA substrates (see Supplementary Information), which indicates that these structurally similar proteins may all share similar biochemical functions. Exogenous dsRNAs can affect gene function in early mouse embryos[18], and our results suggest that this regulation may be accomplished by evolutionarily conserved RNAi machinery.

In addition to RNase III and helicase motifs, searches of the PFAM database indicate that each Dicer family member also contains a PAZ domain (see Supplementary Information)[19,20]. This sequence was defined on the basis of its conservation in the Zwille/ARGONAUTE/Piwi family that has been implicated in RNAi by mutations *in C. elegans* (Rde-1)[9] and *Neurospora* (Qde-2)[10]. Although the function of this domain is unknown, it is notable that this region of homology is restricted to two gene families that participate in dsRNA-dependent silencing. Both the ARGONAUTE and Dicer families have also been implicated in common biological processes, namely the determination of stem-cell fates. A hypomorphic allele of *carpel factory*, a member of the Dicer family in *Arabidopsis*, is characterized by increased proliferation in floral meristems[16]. This phenotype and a number of other characteristic features are also shared by *Arabidopsis ARGONAUTE* (*ago1-1*) mutants[21] (C. Kidner and R. Martiennsen, personal communication). These genetic analyses provide evidence that RNAi may be more than a defensive response to unusual RNAs, but may also have integral functions in the regulation of endogenous genes.

With the identification of Dicer as a potential catalyst of the initiation step of RNAi, we have begun to unravel the biochemical basis of this unusual mechanism of gene regulation. It is now important to determine whether the conserved family members from other organisms, particularly mammals, also have a function in dsRNA-mediated gene regulation.

Note added in proof: Yang *et al.*[22] have recently presented evidence that guide RNAs are derived directly from dsRNA in *Drosophila* embryos. Fagard *et al.*[23] have recently shown that *Arabidopsis* Ago1 is involved in PTGS. □

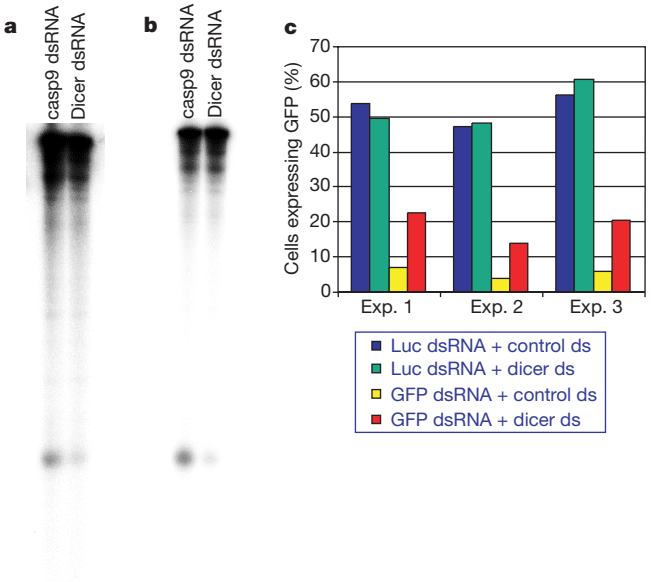

Figure 3 Dicer participates in RNAi. **a**, *Drosophila* S2 cells transfected with dsRNAs corresponding to the two *Drosophila* Dicers (CG4792 and CG6493) or control dsRNA corresponding to murine caspase-9 (casp9). Cytoplasmic extracts of these cells were tested for Dicer activity. Transfection with Dicer dsRNA reduces activity in lysates 7.4-fold. **b**, Dicer-1 antiserum (CG4792) used to prepare immunoprecipitates from S2 cells (treated as above). Dicer dsRNA reduces the activity of Dicer-1 6.2-fold. **c**, GFP expression of co-transfected cells. Three independent experiments were quantified by FACS. A comparison of the relative percentage of GFP-positive cells is shown for control (GFP plasmid plus luciferase dsRNA) or silenced (GFP plasmids plus GFP dsRNA) populations in cells that had previously been transfected with either control (caspase-9) or Dicer dsRNAs.

Methods

Plasmid constructs

A full-length complementary DNA encoding Drosha was obtained by polymerase chain raaction (PCR) from an expressed sequence tag sequenced by the Berkeley *Drosophila* genome project. The T7 epitope-tag was added to the N terminus of each cDNA by PCR, and the tagged cDNAs were cloned into pRIP—a retroviral vector designed specifically for expression in insect cells (E. B., unpublished observations). In this vector, expression is driven by the *Orgyia pseudotsugata* IE2 promoter (Invitrogen). As no cDNA was available for CG4792/Dicer, a genomic clone was amplified from a bac (bacterial artificial chromosome) (BACR23F10; obtained from the BACPAC Resource Center in the Deptartment of Human Genetics at the Roswell Park Cancer Institute). We added a T7 epitope tag at the N terminus of the coding sequence during amplification. We isolated the human *DICER* gene from a cDNA library prepared from HaCaT cells (G.J.H., unpublished observations). A T7-tagged version of the complete coding sequence was cloned into pCDNA3 (Invitrogen) for expression in human cells (LinX-A).

Cell culture and extract preparation

We cultured S2 cells at 27 °C in 5% CO_2 in Schneider's insect media supplemented with 10% heat-inactivated fetal bovine serum (Gemini) and 1% antibiotic–antimycotic solution (Gibco BRL). Cells were collected for extract preparation at 10^7 cells per ml. The cells were washed in PBS and resuspended in a hypotonic buffer (10 mM HEPES pH 7.0, 2 mM $MgCl_2$ and 6 mM β-mercaptoethanol) and lysed. We centrifuged cell lysates at 20,000*g* for 20 min. We stored extracts at −80 °C. We reared *Drosophila* embryos in fly cages by standard methodologies and collected them every 12 h. We dechorionated the embryos in 50% chlorox bleach and washed them thoroughly with distilled water. Lysis buffer (10 mM Hepes, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM EGTA, 10 mM β-glycerophosphate, 1 mM dithiothreitol (DTT) and 0.2 mM PMSF) was added to the embryos, and extracts were prepared by homogenization in a tissue grinder. Lysates were centrifuged for 2 h at 200,000*g*, and were frozen at −80 °C. LinX-A cells, a highly transfectable derivative of human 293 cells (L. Xie and G.J.H., unpublished observations) were maintained in DMEM/10% FCS.

Transfections and immunoprecipitations

We transfected S2 cells using a calcium phosphate procedure essentially as described[6]. Transfection rates were about 90%, as monitored in controls using an *in situ* β-galactosidase assay. We also transfected LinX-A cells by calcium phosphate co-precipitation. For immunoprecipitations, cells (~5×10^6 per immunoprecipitate) were

transfected with various clones, and lysed 3 d later in immunoprecipitate buffer (125 Mm KOAc, 1 mM MgOAc, 1 mM $CaCl_2$, 5 mM EGTA, 20 mM HEPES pH 7.0, 1 mM DTT and 1% Nonidet P40 plus complete protease inhibitors (Roche)). We centrifuged lysates for 10 min at 14,000*g*, and then added supernatants to T7 antibody-agarose beads (Novagen). We performed antibody binding for 4 h at 4 °C. Beads were centrifuged and washed three times in lysis buffer, and once in reaction buffer. The Dicer antiserum was raised in rabbits using a keyhole limpet haemocyanin-conjugated peptide corresponding to the C-terminal eight amino acids of *Drosophila* Dicer-1 (CG4792).

Cleavage reactions

Templates to be transcribed to dsRNA were generated by PCR with forward and reverse primers, each containing a T7 promoter sequence. RNAs were produced using Riboprobe kits (Promega) and were uniformly labelled during the transcription reaction with ^{32}P-labelled UTP. Single-stranded RNAs were purified from 1% agarose gels. For cleavage of dsRNA, 5 μl of embryo or S2 extracts were incubated for 1 h at 30 °C with dsRNA in a reaction containing 20 mM HEPES pH 7.0, 2 mM magnesium acetate, 2 mM DTT, 1 mM ATP and 5% Superasin (Ambion). Immunoprecipitates were treated similarly, except that a minimal volume of reaction buffer (including ATP and superasin) and dsRNA were added to beads that had been washed in reaction buffer. For ATP depletion, *Drosophila* embryo extracts were incubated for 20 min at 30 °C with 2 mM glucose and 0.375 U of hexokinase (Roche), before the addition of dsRNA.

Northern and western analysis

Total RNA was prepared from *Drosophila* embryos (0–12 h), from adult flies and from S2 cells using Trizol (Lifetech). We isolated mRNA by affinity selection using magnetic LIGO-dT beads (Dynal). RNAs were electrophoresed on denaturing formaldehyde/agarose gels, blotted and probed with randomly primed DNAs corresponding to Dicer. For western analysis, T7-tagged proteins were immunoprecipitated from whole-cell lysates in immunoprecipitate buffer using agarose-conjugated anti-T7 antibody. Proteins were released from the beads by boiling in Laemmli buffer, and were separated by 8% SDS–polyacrylamide gel electrophoresis. After transfer to nitrocellulose, proteins were visualized using an HRP-conjugated anti-T7 antibody (Novagen) and chemiluminescent detection (Supersignal, Pierce).

RNAi of Dicer

Drosophila S2 cells were transfected either with a dsRNA corresponding to mouse caspase-9 or with a mixture of two dsRNAs corresponding to *Drosophila* Dicer-1 and Dicer-2 (CG4792 and CG6493). Two days after the initial transfection, cells were again transfected with a mixture containing a GFP expression plasmid and either luciferase dsRNA or GFP dsRNA as described[6]. Cells were assayed for Dicer activity or fluorescence 3 d after the second transfection. Quantification of fluorescent cells was done on a Coulter EPICS cell sorter, after fixation. Control transfections indicated that Dicer activity was not affected by the introduction of caspase-9 dsRNA.

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A model for SOS-lesion-targeted mutations in *Escherichia coli*

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The UmuD′$_2$C protein complex (*Escherichia coli* pol V)[1–3] is a low-fidelity DNA polymerase (pol) that copies damaged DNA in the presence of RecA, single-stranded-DNA binding protein (SSB) and the β,γ-processivity complex of *E. coli* pol III (ref. 4). Here we propose a model to explain SOS-lesion-targeted mutagenesis, assigning specific biochemical functions for each protein during translesion synthesis. (SOS lesion-targeted mutagenesis occurs when pol V is induced as part of the SOS response to DNA damage and incorrectly incorporates nucleotides opposite template lesions.) Pol V plus SSB catalyses RecA filament disassembly in the 3′ to 5′ direction on the template, ahead of the polymerase, in a reaction that does not involve ATP hydrolysis. Concurrent ATP-hydrolysis-driven filament disassembly in the 5′ to 3′ direction results in a bidirectional stripping of RecA from the template strand. The bidirectional collapse of the RecA filament restricts DNA synthesis by pol V to template sites that are proximal to the lesion, thereby minimizing the occurrence of untargeted mutations at undamaged template sites.

Lesions that block DNA replication persist in prokaryotic and eukaryotic cells despite the presence of base excision, nucleotide excision and postreplication repair[5]. A group of DNA polymerases have been discoverd (the UmuC/DinB/Rad30/Rev1 superfamily) whose function is to copy DNA template lesions[6]. The presence of *E. coli* pol V (UmuD′$_2$C) is essential for SOS-induced mutagenesis[5]. However, pol V cannot catalyse translesion synthesis (TLS) by itself; it requires the presence of RecA and single-stranded-DNA binding protein (SSB), and is stimulated by β-sliding clamp in a 'mutasomal' complex[7] (pol V Mut) to copy replication-blocking lesions[4].