

TWNK Substrate Context Separates RNA:DNA Exchange from Replication Claims

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Abstract

Background: TWNK encodes the mitochondrial replicative helicase Twinkle, also known historically as C10orf2 or PEO1. Recent public primary studies span RNA:DNA binding and annealing, DNA strand exchange, real-time DNA unwinding and rewinding, and mouse D-loop replication endpoints. Treating those endpoints as interchangeable can overstate what purified substrate assays show.

Objective: This working paper asks whether public TWNK/Twinkle primary studies separate RNA:DNA binding and annealing/strand-exchange evidence from canonical DNA-unwinding, real-time pause/rewinding, and in vivo D-loop replication endpoints strongly enough to prevent substrate-context overreach.

Methods: Four archived full-text primary records and one current single-molecule comparator were source-coded. Endpoint classes were RNA:DNA binding/annealing/exchange, DNA strand exchange/unwinding, real-time loading/unwinding/pause/rewinding, mouse D-loop/complete mtDNA replication, and smFRET DNA-unwinding context. A portable pilot counted endpoint vocabulary in the four archived primary texts. Quantitative summaries report source-study denominators, repository-file counts and vocabulary counts only; no pooled biological effect, sampling confidence interval or clinical estimate was performed.

Results: Five public primary records were included. The pilot over four archived texts counted 398 RNA terms, 527 DNA-unwinding terms, 153 annealing terms, 173 strand-exchange terms, 73 replication-synthesis terms and 109 single-molecule terms. The Plaza 2025 repository resolved to three public bitstreams: two analysis notebooks and one source-data workbook. The endpoint matrix remained separable across RNA:DNA biochemical activity, DNA exchange/unwinding, optical-tweezers kinetics and mouse replication endpoints. Missing same-condition RNA:DNA exchange plus cellular mtDNA replication/deletion readouts remained unavailable rather than inferred.

Conclusions: Public TWNK evidence supports a substrate-context claim ceiling, not a validated new repair or treatment mechanism. RNA:DNA biochemical findings can motivate follow-on replication-fork and compartment tests, but they should not be cited as direct evidence of cellular repair, mtDNA-deletion prevention, tissue rescue or patient-relevant benefit without paired functional and molecular readouts under the same TWNK perturbation.

Keywords: TWNK; Twinkle helicase; RNA:DNA hybrid; strand exchange; mitochondrial DNA replication; substrate context

1 Introduction

TWINK encodes Twinkle, the human mitochondrial replicative helicase. TWINK aliases C10orf2 and PEO1 identify the same gene, but Twinkle is not POLG and does not provide polymerase or proofreading activity. The selected question is therefore restricted to TWINK/Twinkle substrate and assay interpretation.

The unresolved bottleneck is a substrate and compartment mismatch. Singh et al. reported RNA binding, RNA:DNA annealing and RNA-driven strand-exchange activities in purified Twinkle reactions [1]. Sen, Patel and Patel previously reported DNA strand exchange, branch migration and unwinding activities for TWINKLE [2]. Milenkovic et al. showed that mouse Twinkle is required for nascent D-loop synthesis and complete mtDNA replication [3]. Plaza-G A et al. reported real-time loading, unwinding, pause occupancy and rewinding kinetics for human Twinkle and deposited public notebooks and a source-data workbook [4]. Wang et al. add a current single-molecule FRET comparator for TWINK DNA unwinding and mtSSB effects [5].

The strongest alternative is that these studies simply extend one broad multifunctional Twinkle story, making a separate substrate-context analysis unnecessary. The competing interpretation is plausible because Twinkle can bind, unwind, anneal and exchange nucleic-acid substrates in multiple purified systems. The discriminating question is whether RNA:DNA biochemical observations, DNA exchange/unwinding observations, optical-tweezers pause/rewinding observations and mouse replication endpoints carry different claim ceilings under the same public source set.

This working paper differs from prior Mito Map TWINK renewal-capacity work, which gated build-and-clear mitochondrial quality-control interpretation by intact mtDNA renewal capacity rather than by nucleic-acid substrate class [6]. The present contribution is a source-bound endpoint map and claim-boundary rule. It does not copy another paper's results, does not imply that another paper validates this analysis, and does not infer clinical benefit.

2 Methods

The literature search was refreshed on 2026-10-13 using PubMed, PubMed Central, publisher pages, Nature Communications, the IMDEA Nanociencia repository record at <https://hdl.handle.net/20.500.12614/3946>, and current web search for TWINK, Twinkle, C10orf2, PEO1, RNA:DNA, R-loop, annealing, strand exchange, unwinding, optical tweezers, smFRET, D-loop replication, mtSSB and POLG context. Public primary records were eligible when they directly measured TWINK/Twinkle RNA/DNA substrate activity, DNA exchange/unwinding, single-molecule kinetics or in vivo replication endpoints. Review-only sources, generic mitochondrial concepts, POLG-only records, patient inputs, therapeutic rankings and platform simulations were excluded.

The Plaza 2025 data/code record was inspected directly through its repository handle and DSpace API. The record exposed three downloadable original bitstreams: kymotracker_c_traps.ipynb (698249 bytes), Photon_count_analysis_c_trap.ipynb (1335517 bytes), and Source Data File_Plaza et al.xlsx (6757342 bytes). This inspection demonstrates access feasibility and file identity. The present paper does not reanalyze raw traces and does not treat source-data figures as independent raw traces.

A machine-readable evidence and assumption ledger is provided in Supplementary File S1, with a readable companion in Supplementary File S2. The ledger records exact manuscript anchors, source identifiers, evidence excerpts, source-version hashes, actual check outcomes and unresolved assumptions. It is not a proof of biological truth, clinical validity or therapeutic efficacy.

Each source was coded for organism, biological system, TWINK/Twinkle construct or genotype context, substrate, assay condition, endpoint class, independent unit, nested technical observations, uncertainty convention and statistical or comparison method where the source reported them. Missing source metadata were coded as unavailable. Purified protein reactions, optical-tweezers activities, smFRET traces and mouse tissues were not treated as interchangeable biological replicates.

Endpoint classes were RNA:DNA binding/annealing/exchange, DNA strand exchange/unwinding, single-molecule loading/unwinding/pause/rewinding, mouse D-loop/complete mtDNA replication, and current smFRET DNA-unwinding comparator evidence. The primary analysis was a public source comparison. The portable pilot counted endpoint vocabulary in the four archived primary texts and wrote source-data.json, analysis-summary.json and the source-coded evidence table.

The main functional readout was the reported TWINK/Twinkle nucleic-acid activity or replication endpoint in each source. The orthogonal molecular or compartment readout was the matching substrate, assay system, tissue or same-condition functional context needed to transfer interpretation. Under the selected concept, endpoint classes remain separable and require paired functional plus molecular or compartment readouts before RNA:DNA biochemical activity is used as cellular replication, deletion or rescue evidence. Under the strongest alternative, those classes collapse into a generic multifunctionality statement that does not change citation or experiment-design decisions.

No pooled effect size, confidence interval or significance test was calculated because this analysis tests source separability and claim boundaries, not a shared biological effect. Sensitivity checks were labeled as decision-changing, robustness or exploratory in analysis-summary.json. Vocabulary counts are source-text denominators, not experimental n values.

3 Results

The evidence step first separates endpoint classes, then asks what interpretation changes under the same source conditions.

3.1 Evidence synthesis

The source set contains five public primary records: four archived full texts and one current single-molecule comparator. Singh et al. measured RNA and RNA:DNA substrate activities in purified Twinkle reactions [1]. Sen, Patel and Patel measured DNA strand exchange and unwinding in purified TWINKLE reactions [2]. Milenkovic et al. measured Twinkle knockout consequences for mouse D-loop and complete mtDNA replication [3]. Plaza-G A et al. measured real-time human Twinkle loading, unwinding, pause occupancy and rewinding in biochemical and optical-tweezers systems [4]. Wang et al. measured single-molecule DNA-unwinding pausing and mtSSB effects by smFRET [5].

Table 1. Source-coded evidence and interpretive limits for the TWINK substrate-context analysis.

Figure 1 summarizes the executed workflow from public source retrieval through endpoint coding, unit mapping, sensitivity checks and claim-ceiling synthesis.

The portable pilot over the four archived source texts counted 398 RNA terms, 527 DNA-unwinding terms, 153 annealing terms, 173 strand-exchange terms, 73 replication-synthesis terms and 109 single-molecule terms. These values are vocabulary counts across public source texts. They are not biological sample sizes, effect estimates, protein preparations or sampling confidence intervals.

The unit of analysis for the manuscript claim is the independent source study and endpoint class. The independent source-study count was five for the full evidence table, four for the archived-text pilot, and the repository-file count was three for the Plaza bitstream

inspection. Nested observations include purified reaction replicates, gel time courses, mouse tissue measurements, optical-tweezers activities and smFRET traces as reported by the original authors. Metadata that were not present in the source text or inspected repository record were labeled unavailable rather than inferred.

No selected public source measured RNA:DNA exchange and cellular mtDNA replication or deletion outcomes under the same TWNK perturbation with matched controls. That missing paired readout is decision-changing. RNA:DNA biochemical activity can motivate follow-on replication-fork experiments, but it should not be cited as direct evidence of cellular repair, deletion prevention, tissue rescue or clinical benefit.

Figure 2 shows the concept boundary. RNA:DNA assays, DNA helicase assays, real-time DNA kinetics and in vivo D-loop replication are connected by TWNK biology, but the permitted inference remains substrate- and assay-specific unless paired functional and molecular or compartment readouts are available.

3.2 Evidence-coded sensitivity analysis

The decision-changing class-collapse check asked whether the selected contribution survives if RNA:DNA, DNA unwinding,

real-time kinetics and D-loop replication are treated as one endpoint. It does not. The contribution depends on preserving their non-equivalence.

The robustness check added the current Wang et al. smFRET comparator [5]. The comparator supports current single-molecule TWNK DNA-unwinding relevance and ATP-dependent pausing and mtSSB-mediated stabilization of TWINKLE-DNA interactions, but it does not supply same-condition RNA:DNA exchange plus cellular replication or deletion outcomes. The boundary therefore remains.

The exploratory vocabulary-count check did not recover the manually coded endpoint classes. Its unvalidated keyword heuristic assigned Singh, Sen and Plaza to the same RNA:DNA label and Milenkovic to a source-context label, despite their different assays. Shared terminology, incidental mentions and source references confound classification. The reported term counts are reproducible inventory values only; endpoint distinctions in Table 1 depend on direct reading of the primary studies, not on this classifier. The failed classification is a measurement limitation and supplies no independent validation of the interpretation rule.

Table 1. Source-coded evidence and interpretive limits for the TWNK substrate-context analysis.

ID	Model and perturbation	Key finding	Interpretive limitation
[1]	Purified human Twinkle RNA/RNA:DNA biochemical assays; independent source-study count 1; reactions/titrations are technical observations where reported.	Reports RNA binding, RNA:DNA annealing and RNA-driven strand exchange, with N = 2 examples for selected binding fits and ±SE from fitting where stated.	Physiological role and cellular consequences remain unresolved; purified short-substrate assays do not establish repair, deletion prevention, tissue rescue or patient benefit.
[2]	Purified human TWINKLE DNA strand-exchange/unwinding assays; independent source-study count 1; repeated gel experiments reported at least twice.	Reports coupled unwinding, annealing, strand exchange and branch migration in DNA substrates.	DNA exchange and unwinding cannot be treated as RNA:DNA substrate transfer or as in vivo replication/deletion evidence.
[3]	Mouse Twinkle knockout heart and skeletal-muscle context; independent source-study count 1; animal/tissue counts reported by age and genotype where available.	Reports embryonic requirement, heart/skeletal-muscle mtDNA depletion, impaired D-loop signal and complete mtDNA replication dependence.	Knockout mouse replication endpoints are not equivalent to purified RNA:DNA exchange, variant heterozygosity, overexpression or patient benefit.
[4]	Purified human Twinkle optical-tweezers and biochemical assays; independent source-study count 1; activities/units reported by condition.	Reports real-time loading, unwinding, pause occupancy and rewinding, and a public repository with two notebooks and one source-data workbook.	The source addresses DNA kinetics, not RNA:DNA exchange or same-condition cellular deletion/rescue outcomes.
[5]	Single-molecule FRET TWNK DNA-unwinding comparator; independent source-study count 1; trace categories and ATP/mtSSB conditions are source-reported.	Reports DNA-unwinding pausing and mtSSB effects in a current single-molecule comparator.	Current DNA-unwinding evidence does not collapse RNA:DNA biochemical activity into cellular replication repair or clinical inference.

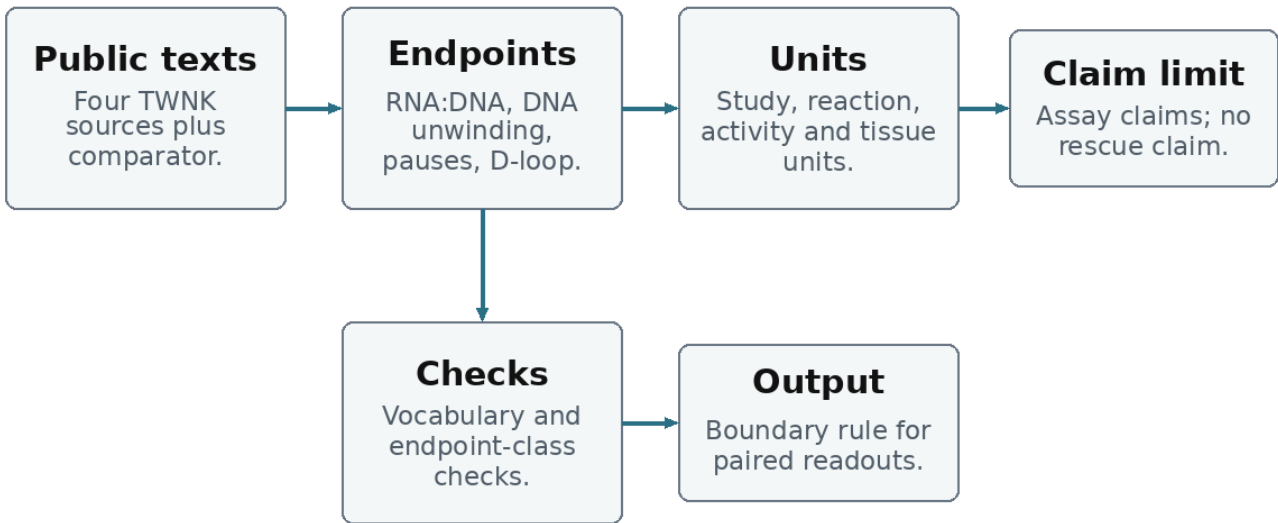


Figure 1. Public source-comparison workflow for the TWNK substrate-context analysis.

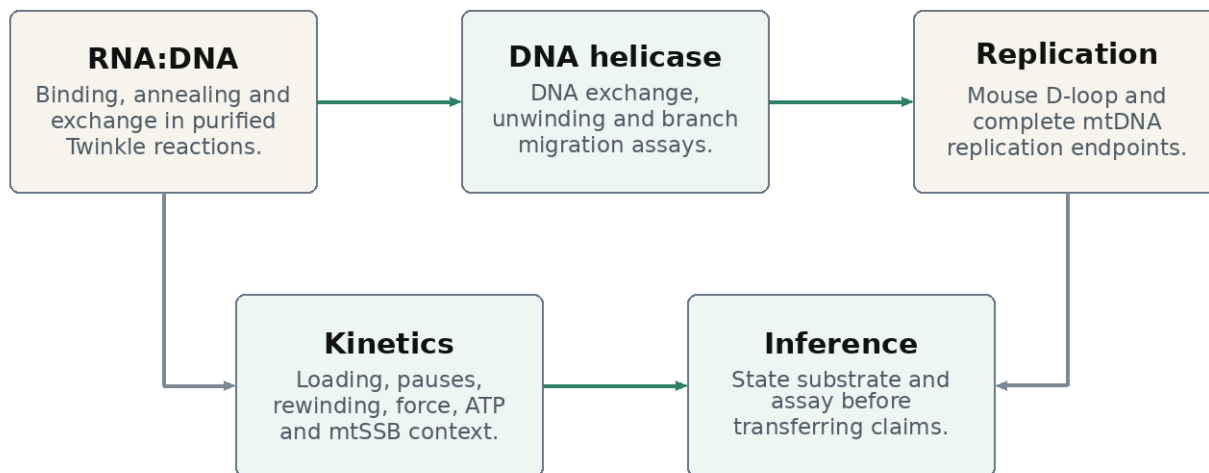


Figure 2. Substrate-context boundary for TWNK RNA:DNA, DNA-unwinding, kinetic and replication endpoints. Arrows organize comparisons and interpretation; they do not establish causal transfer or matched measurements across assays.

4 Discussion

The main result is a claim-ceiling rule. TWNK/Twinkle substrate-context interpretation should name the measured substrate, assay system and endpoint before transferring evidence across RNA:DNA hybrid chemistry, DNA exchange/unwinding, real-time pause/rewinding and in vivo replication. This rule changes a research decision: future TWNK papers and experiments should require paired functional readouts plus orthogonal molecular or compartment readouts before RNA:DNA biochemical findings are used to support cellular repair, deletion prevention or tissue rescue.

The result does not weaken the primary findings of the cited papers. Singh et al. remain the closest RNA:DNA biochemical source [1]. Sen, Patel and Patel remain the closest DNA exchange/unwinding source [2]. Milenkovic et al. remain the closest in vivo D-loop replication source [3]. Plaza-G A et al. remain the closest real-time loading, pause and rewinding kinetics source [4]. Wang et al. provide a current single-molecule comparator for DNA-unwinding pausing [5]. The present contribution is the cross-source interpretation boundary among those endpoint classes.

Ordinary context-dependent Twinkle multifunctionality remains a credible alternative; the endpoint inventory alone does not distinguish a new mechanism from that account. Same-condition substrate and compartment readouts are needed to test whether the proposed interpretation rule adds useful discrimination. A source measuring RNA:DNA exchange and cellular mtDNA replication or deletion outcomes under the same TWNK perturbation, with source organism, construct, force or assay condition, mtSSB/POLG context, tissue or developmental stage, filtering and censoring rules, and independent experimental units preserved, would materially weaken the strict boundary.

The analysis also protects TWNK specificity. No POLG polymerase activity, POLG proofreading, mtDNA repair validation, cell-survival claim, clinical rescue, treatment sequence or safe-overexpression inference follows from this public synthesis. TWNK dose, mtDNA abundance and replication activity may differ by tissue and developmental state, and those differences remain outside the current source-comparison evidence.

5 Limitations

This working paper is a public source-comparison and vocabulary-pilot analysis. It is not a new biochemical experiment, raw-trace reanalysis, animal-level reanalysis, patient study, registered systematic review, meta-analysis or clinical guidance document.

The source systems are heterogeneous. Purified Twinkle reactions, gel assays, optical tweezers, smFRET and mouse knockout tissues differ by construct, substrate, ATP, salt, mtSSB/POLG context,

force, acquisition rate, filtering, censoring, developmental stage and endpoint definition. They should not be pooled as biological replicates.

The Plaza repository inspection established the existence and identity of public notebooks and a source-data workbook, but this paper did not download and reprocess all raw laboratory traces or author-held metadata. Missing trace, protein-preparation or same-condition cellular readouts are therefore bounded as unavailable in the inspected public record, not as proof that the original laboratories lack those data.

6 Conclusions

Public TWNK/Twinkle evidence supports substrate-specific interpretation across RNA:DNA biochemical activity, DNA exchange/unwinding, real-time DNA kinetics and mouse D-loop replication. It does not support a stronger cellular repair, deletion-prevention, tissue-rescue or patient-benefit claim without paired same-condition functional and molecular or compartment readouts. The useful next step is not broader wording; it is a matched TWNK perturbation design that measures substrate class, replication intermediates, RNA:DNA hybrids, mtDNA abundance or deletion burden, and independent units in the same system.

Declarations

Data and code availability. The repository deposit includes the manuscript source, evidence table, source data, analysis code, figures, measured figure geometry, biological-audit supplements, layout reports, QA record, Gemini review and checksums needed to replay the table, counts, figures and artifacts. Private replay inputs are retained locally and excluded from public manifests. Manuscript-quality and concept-impact grades are available at <https://precisionmito.com/paper-grades>.

Source verification. The biological audit is a manuscript-integrity ledger, not proof of biological truth or clinical efficacy. Corpus anchors and archived normalized source files were replayed against recorded hashes; UNKNOWN or NOT_RUN biological interpretation statuses remain visible.

Ethics statement. This literature synthesis used no identifiable patient data and required no new human-participant or animal research.

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Competing interests. The author reports no competing interests for this working paper.

Author contributions. Andrew Friedl conceived the project, directed the analysis, reviewed the evidence package, and approved the manuscript for repository review.

AI assistance. AI tools assisted literature synthesis, drafting, data visualization, formatting and QA preparation. Gemini independently reviewed the exact rendered PDF when artifact-bound review was available. Andrew Friedl remains solely responsible for the content and is the sole author.

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