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RESEARCH ARTICLE

TRIP/NOPO E3 ubiquitin ligase promotes ubiquitylation of DNA polymerase η

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ABSTRACT

We previously identified a *Drosophila* maternal effect-lethal mutant named '*no poles*' (*nopo*). Embryos from *nopo* females undergo mitotic arrest with barrel-shaped, acentrosomal spindles during the rapid cycles of syncytial embryogenesis because of activation of a Chk2-mediated DNA checkpoint. NOPO is the *Drosophila* homolog of human TNF receptor associated factor (TRAF)-interacting protein (TRIP), which has been implicated in TNF signaling. NOPO and TRIP contain RING domains closely resembling those of known E3 ubiquitin ligases. We herein sought to elucidate the mechanism by which TRIP/NOPO promotes genomic stability by performing a yeast two-hybrid screen to identify potential substrates/interactors. We identified members of the Y-family of DNA polymerases that facilitate replicative bypass of damaged DNA (translesion synthesis) as TRIP interactors. We show that TRIP and NOPO co-immunoprecipitate with human and *Drosophila* Polη, respectively, from cultured cells. We generated a null mutation in *Drosophila Polη* (*dPolη*) and found that *dPolη*-derived embryos have increased sensitivity to ultraviolet irradiation and exhibit *nopo*-like mitotic spindle defects. *dPolη* and *nopo* interact genetically in that overexpression of *dPolη* in hypomorphic *nopo*-derived embryos suppresses *nopo* phenotypes. We observed enhanced ubiquitylation of Polη by TRIP and NOPO E3 ligases in human cells and *Drosophila* embryos, respectively, and show that TRIP promotes hPolη localization to nuclear foci in human cells. We present a model in which TRIP/NOPO ubiquitylates Polη to positively regulate its activity in translesion synthesis.

KEY WORDS: *Drosophila*, Cell cycle, Embryogenesis, DNA damage, Xeroderma pigmentosum, E3 ubiquitin ligase

INTRODUCTION

The early embryonic development of organisms such as *Drosophila melanogaster*, *Xenopus laevis*, *Danio rerio* and *Caenorhabditis elegans* are characterized by rapid progression through the cell cycle (Budirahardja and Gönczy, 2009; O'Farrell et al., 2004). In *Drosophila*, early embryonic cell cycles consist of 13 oscillating rounds of DNA replication and mitosis without intervening gap phases or cytokinesis. Cell-cycle regulators are provided maternally and are slowly depleted until the midblastula transition. *meiotic 41* (*mei-41*) and *grapes* (*grp*), which encode *Drosophila* homologs of ATM and Rad3-related (ATR) and Checkpoint 1 (Chk1) kinases, respectively, are essential effectors of a DNA damage/replication checkpoint pathway required to slow late syncytial cell cycles (11-13) in order to introduce a G2 phase (Fogarty et al., 1994; Sibon et al., 1999; Sibon et al., 1997).

The capacity of cells to respond to potentially detrimental DNA damage is of crucial importance for efficient and accurate replication of the genetic material. Most eukaryotic cells employ highly regulated responses to damaged DNA involving ATR and ataxia telangiectasia mutated (ATM), and their effector kinases Chk1 and Checkpoint 2 (Chk2), which trigger signaling cascades to respond to and repair damage (Abraham, 2001; Sancar et al., 2004). During the rapid divisions of syncytial embryogenesis, however, there may be insufficient time for canonical checkpoints to facilitate DNA repair (O'Farrell et al., 2004). Because accumulated DNA damage may block progression of replication forks, cells possess mechanisms to temporarily tolerate DNA damage until it can be repaired (Sale et al., 2012; Waters et al., 2009).

Translesion synthesis (TLS), which promotes completion of DNA replication when the replication fork encounters damage, is one mechanism used by the cell to bypass damage during S phase (Sale et al., 2012; Waters et al., 2009). This process is initiated by proliferating cell nuclear antigen (PCNA) mono-ubiquitylation, which triggers recruitment of specialized DNA polymerases to the replication fork (Freudenthal et al., 2010; Kannouche et al., 2004). These polymerases include Polζ (also referred to as Rev3), a member of the B-family of DNA polymerases, and members of the Y-family of DNA polymerases: Rev1, Polι, Polη and Polκ (Kannouche and Lehmann, 2006; Lehmann et al., 2007; Sale et al., 2012; Shaheen et al., 2010). Mutation of human Polη results in a variant form of xeroderma pigmentosum (XP-V), a disease characterized by increased UV sensitivity and susceptibility to skin cancers (Kannouche et al., 2001; Kannouche and Stary, 2003; Masutani et al., 1999).

Three members of the Y-family of DNA polymerases are conserved in *Drosophila*: dPolη, dPolι and dRev1 (Ishikawa et al., 2001). dPolη and dPolι are functional translesion polymerases *in vitro* (Ishikawa et al., 2001). *dPolη* larvae exhibit increased sensitivity to UV irradiation, and homologous recombination is impaired in *dPolη* flies (Kane et al., 2012). Germline knockdown of *polh-1*, the *C. elegans Polη* homolog, causes increased sensitivity of early embryos to UV irradiation (Ohkumo et al., 2006). Recent studies suggest that POLH-1 functions in *C. elegans* to keep the rapid cell cycles of early embryogenesis on schedule (Holway et al., 2006; Kim and Michael, 2008).

We previously described a *Drosophila* maternal effect-lethal mutant named '*no poles*' (*nopo*) (Merkle et al., 2009). Embryos from *nopo* females undergo mitotic arrest during the rapid S-M cycles of syncytial embryogenesis. Chk2 plays a checkpoint function in the *Drosophila* early embryo with localized activation in

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response to DNA damage or incomplete replication (Sibon et al., 2000; Takada et al., 2003). We found evidence of Chk2 activation in *nopo*-derived embryos, suggesting that NOPO promotes genomic maintenance in early embryos (Merkle et al., 2009). Based on the abnormally short interphase 11 of *nopo*-derived embryos, we hypothesized that they enter mitosis before completing DNA replication, thereby activating Chk2.

nopo, which encodes a RING domain-containing candidate E3 ubiquitin ligase, is the *Drosophila* homolog of the human gene encoding TRAF-interacting protein (TRIP). Tumor necrosis factor receptor (TNFR)-associated factors (TRAFs) are key adaptor molecules in the TNF-signaling pathway that promote cell proliferation, activation, differentiation, and apoptosis (Ha et al., 2009). Whether TRIP plays a role in TNF signaling, however, is unclear (Lee et al., 1997; Regamey et al., 2003).

Although TRIP/NOPO substrates have not been reported to date, mouse TRIP is a functional E3 ligase *in vitro* (Besse et al., 2007). E3 ligases facilitate transfer of ubiquitin from an E2 conjugating enzyme to substrates (Fang and Weissman, 2004). Ubiquitylation plays important roles in many cellular processes, including protein processing, cell-cycle control, chromatin remodeling, DNA repair and membrane trafficking by targeting proteins for destruction by the 26S proteasome, altering subcellular localizations, and/or changing protein-protein interactions (Acconcia et al., 2009; Al-Hakim et al., 2010; Broemer and Meier, 2009; Glickman and Ciechanover, 2002; Hershko, 1997; Le Bras et al., 2011; O'Connell and Harper, 2007).

To elucidate the mechanism by which the TRIP/NOPO E3 ligases promote genomic stability, we performed a yeast two-hybrid screen using human TRIP as bait. We report herein the interaction of human TRIP and *Drosophila* NOPO with members of the Y-family of DNA polymerases in yeast two-hybrid assays and their co-immunoprecipitation from cultured cells. We generated a null allele of *Drosophila Polη* (*dPolη*) and found that *dPolη*-derived embryos are sensitive to UV irradiation and have *nopo*-like mitotic spindle defects. We find that overexpression of *dPolη* in early embryos compensates for partial loss of NOPO. We show that TRIP/NOPO enhances ubiquitylation of human Polη (hPolη) and dPolη in cultured cells and *Drosophila* embryos, respectively, and that TRIP promotes localization of hPolη into nuclear foci in cultured human cells. These findings suggest that TRIP/NOPO plays an important role in positively regulating the DNA damage tolerance activity of Polη during *Drosophila* embryogenesis and in human cells.

RESULTS

Drosophila NOPO and human TRIP are functional homologs

Embryos derived from *Drosophila nopo* females fail to progress to larval stages due to mitotic arrest with barrel-shaped, acentrosomal spindles during the rapid S-M cycles of syncytial embryogenesis (Merkle et al., 2009). We previously demonstrated rescue of *nopo* mutants by using a transgenic line carrying *nopo* genomic sequence (Merkle et al., 2009). Herein we generated a new transgenic line in which *nopo* cDNA was placed under control of the endogenous *nopo* promoter (Fig. 1A). Embryonic expression of this transgene was confirmed by RT-PCR (Fig. 1B). Embryos produced by *nopo* females were rescued by the presence of the *nopo* cDNA transgene, exhibiting a 76% hatch rate (embryo-to-larva transition) compared with 0% and 94% for *nopo* and wild-type females, respectively (Fig. 1C).

To test for functional conservation of human TRIP and *Drosophila* NOPO E3 ubiquitin ligases, we investigated whether transgenic expression of human *TRIP* under control of the endogenous *nopo* promoter could rescue *Drosophila nopo* mutants (Fig. 1A). Embryonic expression of this transgene was confirmed by RT-PCR (Fig. 1B). Fertility was restored to *nopo* females carrying the *TRIP* cDNA transgene, resulting in an 83% hatch rate (Fig. 1C). Mitotic spindles were also restored to a wild-type morphology: whereas 94% of embryos from *nopo* females exhibited abnormal spindle morphology, the frequency was reduced to 22% and 14% in embryos carrying the *nopo* or *TRIP* transgenes, respectively (Fig. 1D,E). These data suggest that human TRIP is a functionally conserved homolog of *Drosophila* NOPO.

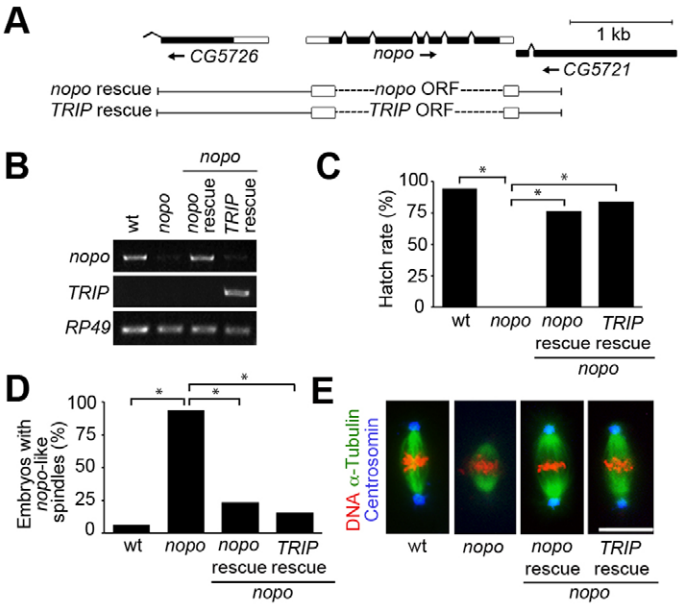

Fig. 1. Transgenic human TRIP expression rescues *Drosophila nopo* mutants. (A) Schematic of the *nopo* gene region (top). The black boxes represent coding regions, white boxes represent 5′ and 3′ untranslated regions (UTRs), and lines represent introns. Transgenic rescue constructs (bottom) contain genomic flanking sequence (lines), 5′- and 3′-UTRs of *nopo* (white boxes), and *nopo* or *TRIP* open reading frame (ORF; dashed lines). (B) RT-PCR confirming nopo or *TRIP* transgenic expression in $nopo^{Exc142}$-derived embryos (0-2 hours). Positive and negative controls: wild-type and $nopo^{Exc142}$-derived embryos, respectively. Loading control: *RP49*. (C) *nopo* or *TRIP* expression restored fertility to $nopo^{Exc142}$ females. (D) *nopo* or *TRIP* expression rescued aberrant spindles of $nopo^{Exc142}$-derived embryos. (E) Embryos stained for Centrosomin (blue), α-tubulin (green) and DNA (red). Representative mitotic spindles in embryos from females of indicated genotypes are shown. Asterisks, $P<0.0001$ (indicated pairwise comparisons). Scale bar: 10 µm. wt, wild type.

TRIP interacts with Y-family DNA polymerases

RING domain-containing E3 ligases bind directly to their substrates to catalyze direct transfer of ubiquitin from an E2 ubiquitin conjugating enzyme (Deshaies and Joazeiro, 2009). To elucidate the role of NOPO in maintaining genomic integrity during early embryonic development in *Drosophila*, we sought to identify potential substrate(s) of the NOPO E3 ubiquitin ligase by performing a yeast two-hybrid screen. Due to a lack of commercially available, high-quality cDNA libraries prepared from *Drosophila* early embryos, we chose to carry out our yeast-two hybrid screen by using TRIP, the human homolog of NOPO, as the bait and a HeLa cell cDNA library as the prey. Sequence analysis revealed that one of the first colonies to appear on selective media in this screen contained a clone encoding a C-terminal fragment of hPolκ, a member of the Y-family of DNA polymerases.

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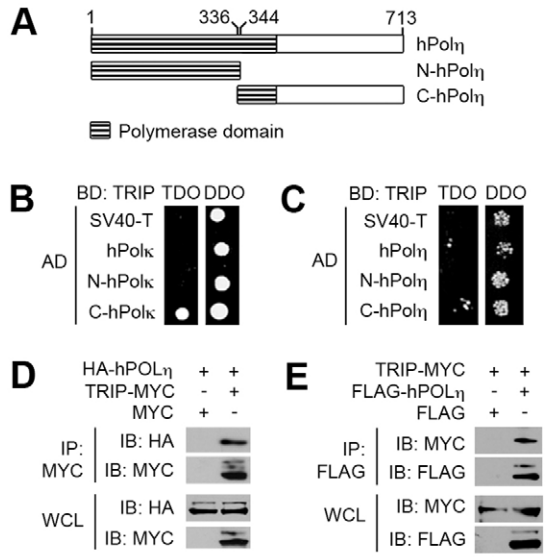

Fig. 2. TRIP interacts with human Y-family DNA polymerases. (A) Schematic representation of full-length and truncated forms of hPolη (prey). (B,C) Yeast two-hybrid assays. Yeast expressing human TRIP (BD, 'bait') and full-length or truncated forms of hPolκ (B) or hPolη (C) (AD, 'prey') were spotted onto selective triple dropout (TDO) media to assess interactions. Plating on double dropout (DDO) media confirmed presence of bait and prey constructs. Negative control: SV40 T-antigen. (D,E) hPolη and TRIP co-immunoprecipitation. (D) HeLa cells were co-transfected with indicated plasmids. TRIP-MYC immunoprecipitates and whole cell lysates were analyzed by immunoblotting. (E) Reciprocal co-IP of FLAG-hPolη complexes from HeLa cells co-transfected as indicated. IB, immunoblotting; IP, immunoprecipitates; WCL, whole cell lysates.

To confirm the interaction of TRIP with hPolκ and to determine if TRIP interacts with other members of the Y-family of DNA polymerases (hPolη, hPolι and hRev1), we performed yeast two-hybrid assays using TRIP as bait with full-length or truncated versions of these polymerases as prey (illustrated for hPolη in Fig. 2A). We found that TRIP interacted with the C-terminal half of hPolκ, but not with the N-terminal half or full-length form of this polymerase (Fig. 2B). Similarly, TRIP interacted with the C-terminal half of hPolη; we also observed interaction between TRIP and the full-length form, but not the N-terminal half, of hPolη (Fig. 2C). These data are consistent with previous reports demonstrating the existence of several protein-protein interaction domains in the C-terminal regions of Y-family DNA polymerases (Waters et al., 2009). Our results for hPolι and hRev1 were inconclusive (data not shown).

To further verify these interactions, we co-expressed MYC-tagged TRIP and epitope-tagged hPolη and hPolκ (HA- or FLAG-tagged) in HeLa cells for co-immunoprecipitation experiments. We detected the presence of HA-hPolη in TRIP-MYC immunoprecipitates; reciprocally, we detected the presence of TRIP-MYC in FLAG-hPolη immunoprecipitates, thereby confirming the yeast two-hybrid interaction between TRIP and hPolη in this system (Fig. 2E,F). We were unable, however, to detect co-immunoprecipitation of tagged hPolκ and MYC-TRIP (data not shown). We frequently observed a faint, more slowly migrating form of tagged hPolη on immunoblots of cell lysates; this band may represent a mono-ubiquitylated form of hPolη as reported previously (Bienko et al., 2010).

NOPO interacts with Y-family DNA polymerases

Three of the four members of the human Y-family DNA polymerases are conserved in *Drosophila*: Polη, Polι and Rev1 (Fig. 3A). To determine if NOPO, like its human homolog, interacts with the *Drosophila* Y-family DNA polymerases, we performed yeast two-hybrid assays using NOPO as bait with full-length or truncated versions of each of these polymerases as prey (as illustrated for dPolη in Fig. 3B). We found that NOPO interacted with the C-terminal halves and full-length forms of both dPolη and dPolι; results for dRev1 were inconclusive (Fig. 3C,D; data not shown). To further verify these interactions, we co-expressed MYC-tagged NOPO and HA-tagged dPolη or dPolι in *Drosophila* S2 cells. We detected the presence of HA-dPolη, but not HA-dPolι, in NOPO-MYC immunoprecipitates, thereby confirming the yeast two-hybrid interaction between NOPO and dPolη in this system (Fig. 3E; data not shown).

Drosophila Y-family polymerases are expressed in early embryos

The yeast two-hybrid interactions and co-immunoprecipitations observed between TRIP/NOPO E3 ubiquitin ligases and Y-family DNA polymerases suggested that these proteins might function in a common pathway to preserve genomic integrity. To determine if these polymerases are expressed during *Drosophila* early embryogenesis, we performed RT-PCR using mRNA extracted from 0-2 hour embryos. We found that, like *nopo*, all of the Y-family DNA polymerases are expressed during syncytial embryogenesis (Fig. 4A).

Mutations in hPolη result in a variant form of xeroderma pigmentosum, a disease characterized by an increased incidence of UV-induced skin cancer. Due to the key role of Polη in TLS and its link to human disease, as well as the lack of available mutants to study the functions of the other *Drosophila* Y-family polymerases, we chose to focus the remainder of our study on dPolη. To

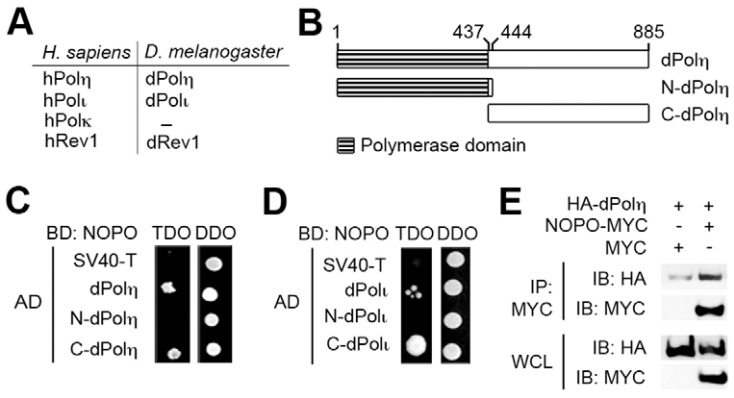

Fig. 3. NOPO interacts with *Drosophila* Y-family DNA polymerases. (A) Conservation of human and *Drosophila* Y-family DNA polymerases. (B) Schematic representation of full-length and truncated forms of dPolη (prey). (C,D) Yeast two-hybrid assays. Yeast expressing NOPO (BD, 'bait') and full-length or truncated forms of dPolη (C) or dPolι (D) (AD, 'prey') were spotted onto selective triple dropout (TDO) media to assess interactions. Plating on double dropout (DDO) media confirmed presence of bait and prey constructs. Negative control: SV40 T-antigen. (E) dPolη and NOPO co-immunoprecipitation. S2 cells were co-transfected as indicated. NOPO-MYC immunoprecipitates and whole cell lysates were resolved by immunoblotting.

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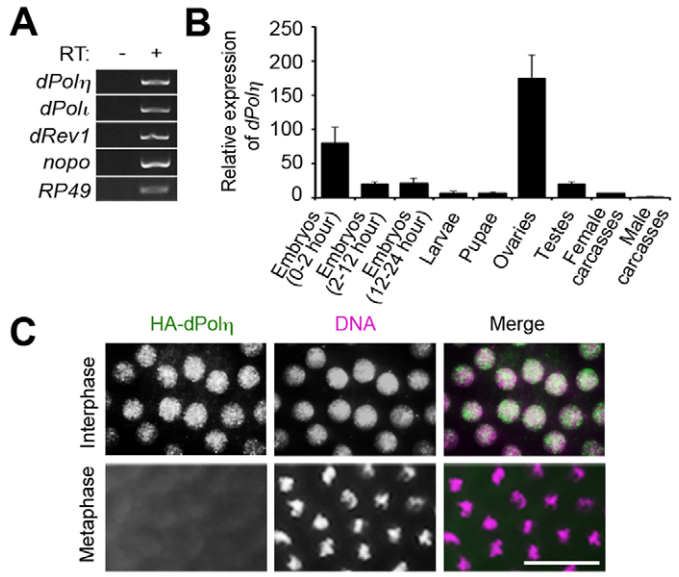

Fig. 4. *Drosophila* Y-family DNA polymerase expression and localization. (A) RT-PCR analysis of *dPolη*, *dPolι* and *dRev1* in embryos (0-2 hour). Positive controls: *RP49* and *nopo*. (B) *dPolη* developmental expression. Mean relative expression (normalized against *RP49*) ± s.e.m. is displayed. (C) Embryos from females carrying *UASp-HA-dPolη* and *nos-Gal4* immunostained for HA (green) and DNA (magenta). HA-dPolη localized to interphase nuclei (top panels) but was undetectable during mitosis (bottom panels). Scale bar: 10 µm.

determine the tissues and developmental stages in which dPolη is expressed, we performed RT-PCR using mRNA isolated from embryos of various stages, larvae, pupae, ovaries, testes, and male and female carcasses. We found that dPolη is most highly expressed in 0-2 hour embryos and ovaries, suggesting that it could potentially interact with NOPO *in vivo* to promote maintenance of genomic integrity during early embryogenesis (Fig. 4B).

To determine the localization of dPolη in early embryos, we generated a transgenic line for expression of HA-tagged full-length dPolη under control of the UASp promoter (Rørth, 1998). Using *nanos-Gal4:VP16* (‘*nos-Gal4*’) for germline expression of this transgene, we performed immunostaining of syncytial embryos and found HA-dPolη to localize within interphase nuclei; no signal was detected on mitotic chromosomes (Fig. 4C). These observations are consistent with the previously reported localization of hPolη in cultured cells (Kannouche et al., 2003; Kannouche and Lehmann, 2006).

dPolη-derived embryos are sensitive to DNA-damaging agents and have *nopo*-like defects

To determine if *dPolη*, like *nopo*, is required during early embryogenesis, we generated a null allele of *dPolη* (*Exc2.15*) via imprecise excision of *P*-element *EY07711* in the 5′ UTR of the *dPolη* gene (Fig. 5A; supplementary material Fig. S1A). Homozygous $dPol\eta^{Exc2.15}$ females are viable and fertile, producing embryos with wild-type hatch rates; similar results were obtained for females carrying $dPol\eta^{Exc2.15}$ in trans to *Df(3L)BSC284,* which uncovers *dPolη* (supplementary material Fig. S1B). This observation is consistent with studies of POLH-1 during *C. elegans* embryogenesis and suggests that functional redundancy among Y-family polymerase members may account for the lack of severe phenotypes in single mutants (Roerink et al., 2012).

Polη is known to specifically bypass UV-induced lesions in yeast and mammals, and dPolη has been shown to synthesize efficiently past cyclobutane pyrimidine dimers *in vitro* (Ishikawa et al., 2001; Johnson et al., 1999; McCulloch et al., 2004; McDonald et al., 1997). We therefore tested the sensitivity of embryos from

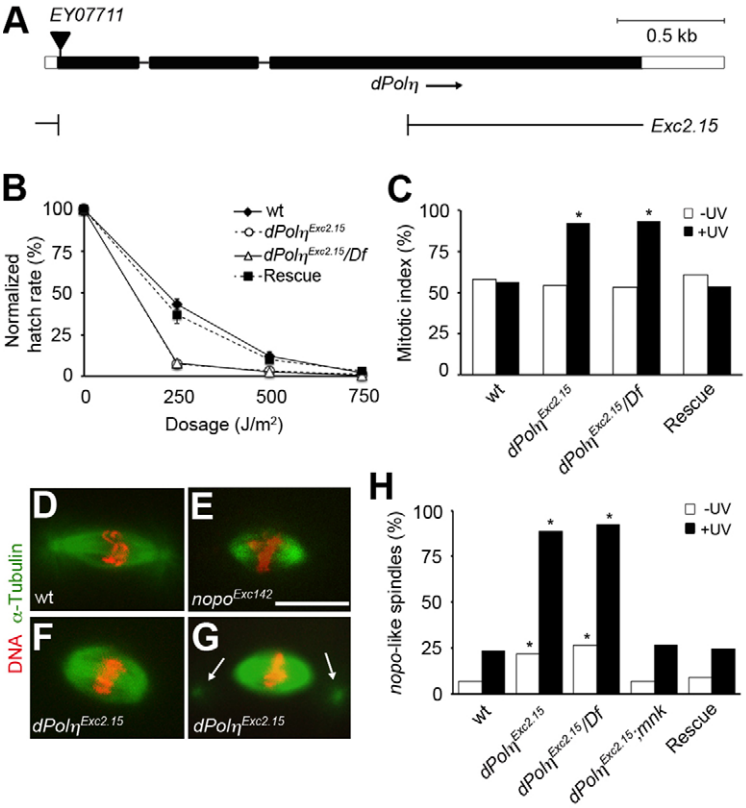

Fig. 5. Increased UV sensitivity and *nopo*-like spindles of *dPolη* mutants. (A) Schematic of *dPolη* gene region. The black boxes represent coding regions, white boxes represent 5′ and 3′ untranslated regions (UTRs), and lines represent introns. $dPol\eta^{Exc2.15}$ (gap represents deleted region) was generated by imprecise excision of *P*-element *EY07711* (triangle). (B) UV sensitivity assay. Hatch rates of UV-irradiated embryos were normalized to untreated controls for each genotype; hatch rates of untreated controls were ~95% (supplementary material Fig. S1; data not shown). Mean values from ≥3 independent experiments are displayed. Error bars, s.d. (C) Quantification of mitotic index in untreated or UV-irradiated embryos. Asterisks, $P<0.0001$ (relative to untreated controls). (D-G) Representative mitotic spindles in embryos stained for α-tubulin (green) and DNA (red). Arrows mark detached centrosomes. (H) Quantification of *nopo*-like spindles in untreated or UV-irradiated embryos. Data are plotted as percentage of embryos with >50% *nopo*-like spindles. Phenotypes presented in C-H were observed throughout syncytial embryogenesis. For bar graphs: asterisks, $P<0.0001$ (compared to untreated or UV-irradiated wild-type control). *Df=Df(3L)BSC284*. Scale bar: 10 µm.

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dPolη$^{Exc2.15}$ females to various doses of UV irradiation. Hatch rates of embryos from both wild-type and *dPolη*$^{Exc2.15}$ females decreased following UV exposure, but *dPolη*$^{Exc2.15}$-derived embryos were significantly more affected; germline expression of HA-dPolη restored a wild-type degree of UV sensitivity to *dPolη*$^{Exc2.15}$-derived embryos (Fig. 5B). Immunostaining revealed a significant increase in mitotic index in response to UV irradiation in *dPolη*$^{Exc2.15}$-derived embryos that was not seen in wild-type embryos; this effect was also rescued by germline expression of HA-dPolη (Fig. 5C). These findings are consistent with previous work showing that a deletion in *dPolη* caused increased sensitivity of third-instar larvae to UV irradiation (Kane et al., 2012).

In contrast to *nopo*-derived embryos, unperturbed *dPolη*$^{Exc2.15}$-derived embryos appeared to progress through syncytial embryogenesis at a normal rate and hatched normally (supplementary material Fig. S1B; Fig. S2). Immunostaining, however, revealed that a majority of spindles in 22% of untreated *dPolη*$^{Exc2.15}$-derived embryos (compared with 5% of wild-type embryos) had *nopo*-like defects (Fig. 5D-H). Upon exposure to 250 J/m^2 UV, this frequency increased to 90% for *dPolη*$^{Exc2.15}$-derived embryos compared with 24% for wild-type embryos (Fig. 2H). Germline expression of HA-dPolη restored the frequency of spindle defects to wild-type levels in *dPolη*$^{Exc2.15}$-derived embryos with or without UV treatment (Fig. 5H).

We previously reported that syncytial embryos from *nopo* females undergo Chk2-mediated mitotic arrest (Merkle et al., 2009). Chk2 is activated in *Drosophila* syncytial embryos in response to mitotic entry with incompletely replicated or damaged DNA (Sibon et al., 2000; Takada et al., 2003). We showed that *nopo* defects could be suppressed by mutation of *maternal nuclear kinase* (*mnk*; *lok* – FlyBase), which encodes the *Drosophila* Chk2 homolog, suggesting that NOPO promotes genomic integrity during early embryogenesis (Abdu et al., 2002; Brodsky et al., 2004; Masrouha et al., 2003; Merkle et al., 2009; Xu et al., 2001). To determine whether Chk2 mediates the mitotic defects of *dPolη*$^{Exc2.15}$-derived embryos, we generated flies mutant for both *dPolη* and *mnk*. The mild *nopo*-like spindle phenotype observed in unchallenged *dPolη*$^{Exc2.15}$-derived embryos was restored to wild-type levels in embryos from double-mutant females; *mnk* also suppressed the spindle abnormalities in UV-irradiated embryos from *dPolη*$^{Exc2.15}$ females (Fig. 5H). These data suggest that dPolη is required for normal progression through mitosis when DNA damage is present during early embryogenesis.

Human Polη is recruited to replication forks and is crucial for S-phase progression in response to hydroxyurea (HU)-induced DNA replication stress (Kane et al., 2012). We therefore tested the sensitivity of *dPolη*$^{Exc2.15}$ larvae to HU. We found that, similar to *mei-41*^{RT1} control larvae, *dPolη*$^{Exc2.15}$ larvae were sensitive to HU treatment, exhibiting a significant reduction in survival compared with expected Mendelian ratios (supplementary material Fig. S3). These results suggest that *dPolη* plays an important role in responding to HU-induced DNA damage.

dPolη overexpression suppresses *nopo* phenotypes

To further explore the possibility of a functional relationship between *nopo* and *dPolη*, we looked for genetic interactions following germline overexpression of HA-dPolη in females homozygous for *nopo*^{SZ3004}, a hypomorphic allele (Merkle et al., 2009). Embryos from *nopo*^{SZ3004} females carrying either *nos-Gal4* or *UASp-HA-dPolη* transgenes exhibited decreased hatch rates of 42% and 44%, respectively, compared with the wild-type hatch rate of 94% (Fig. 6A). Mitotic spindle defects occurred at a frequency of 43% and 48% in embryos from *nopo*^{SZ3004} females carrying either *nos-Gal4* or *UASp-HA-dPolη* transgenes, respectively, compared with a wild-type frequency of 15% (Fig. 6B). Both phenotypes were strongly suppressed in embryos from *nopo*^{SZ3004} females with germline overexpression of HA-dPolη: hatch rates increased to 90%, and the frequency of mitotic spindle defects decreased to 25% (Fig. 6A,B).

Embryos from *nopo*^{SZ3004} females carrying *nos-Gal4* or *UASp-HA-dPolη* transgenes were developmentally delayed with only 13% or 15%, respectively, reaching gastrulation by 5 hours after egg deposition compared with 93% of wild-type embryos (Fig. 6C). Germline overexpression of HA-dPolη in *nopo*^{SZ3004} females suppressed this developmental delay with 84% of embryos reaching gastrulation by 5 hours after egg deposition (Fig. 6C). Taken together, these data suggest that dPolη overexpression can compensate for the loss of *nopo* and that dPolη and NOPO contribute to the same biological process. Conversely, we did not observe enhancement of *nopo*^{SZ3004} phenotypes in *nopo*^{SZ3004} *dPolη* double mutants (data not shown).

TRIP/NOPO E3 ubiquitin ligase enhances Polη ubiquitylation

We considered the possibility that Polη might be a substrate of TRIP/NOPO E3 ubiquitin ligase. Using a cell-based, polyhistidine-ubiquitin assay, we tested whether TRIP overexpression in cultured human cells could promote Polη ubiquitylation (Campanero and Flemington, 1997; Treier et al., 1994). Without TRIP overexpression, we detected a single ubiquitylated species of hPolη, consistent with previous studies demonstrating its mono-ubiquitylation (Fig. 7A) (Bienko et al., 2010). TRIP overexpression

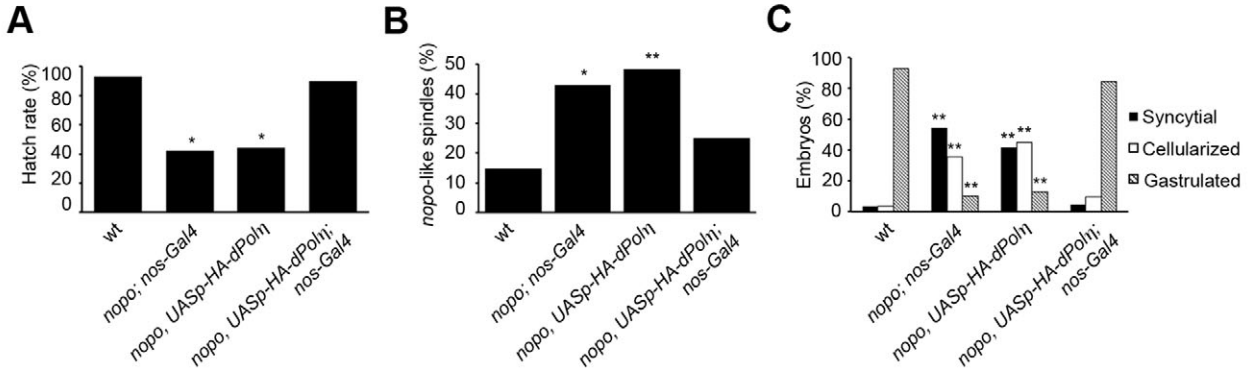

Fig. 6. Suppression of *nopo* by dPolη overexpression. (A) germline *dPolη* overexpression restored fertility to *nopo*^{SZ3004} females. (B,C) Embryos (B, 0-2 hour; C, 3-5 hour) were stained for α-tubulin and DNA. Mitotic spindle abnormalities (B) and early developmental arrest (C) of *nopo*^{SZ3004}-derived embryos were rescued by germline *dPolη* overexpression. Single asterisks, $P<0.001$; double asterisks, $P<0.0001$ (compared to wild type).

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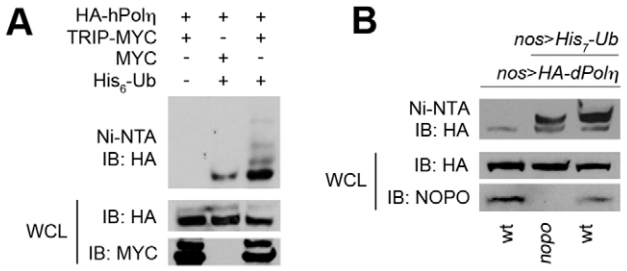

Fig. 7. TRIP/NOPO enhances Polη ubiquitylation. (A) His-ubiquitin assay in HeLa cells. Cells co-transfected as indicated were lysed under denaturing conditions. Ubiquitylated proteins recovered by nickel affinity chromatography (Ni-NTA) and whole cell lysates were analyzed by immunoblotting. (B) His-ubiquitin assay in *Drosophila* embryos. Assays were performed as in A using embryos from wild-type or *nopo*^{Exc142} females with germline expression of HA-dPolη ± His$_7$-ubiquitin (His$_7$-Ub). IB, immunoblotting; WCL, whole cell lysates.

caused further ubiquitylation of HA-hPolη as evidenced by its upward laddering and increased signal intensity in this assay. These data suggest that TRIP promotes hPolη ubiquitylation.

Ubiquitylation of Y-family DNA polymerases has been shown in budding yeast, *C. elegans* and mammalian cells, but not in *Drosophila* (Skoneczna et al., 2007; Kim and Michael, 2008; Jung et al., 2010; Bienko et al., 2010). To determine whether dPolη is ubiquitylated during *Drosophila* embryogenesis, we used an *in vivo*, polyhistidine-ubiquitin assay. We found that HA-dPolη was ubiquitylated in syncytial embryos from wild-type females with germline expression of HA-dPolη and His$_7$-Ub; by contrast, *nopo*^{Exc142}-derived embryos showed a significant, albeit modest, decrease in the level of ubiquitylated dPolη in this assay (Fig. 7B; supplementary material Fig. S4). These data suggest that NOPO promotes ubiquitylation of dPolη during early embryogenesis. Because *nopo*^{Exc142}-derived embryos arrest during syncytial embryogenesis, however, we cannot rule out the possibility that observed changes in dPolη ubiquitylation in *nopo*^{Exc142}-derived embryos might be a secondary effect.

hPolη colocalizes with TRIP in cultured human cells

We previously reported that N-terminally tagged eGFP-TRIP localizes to nuclear puncta during G2 phase in transfected HeLa cells (Merkle et al., 2009). Another group reported that endogenous TRIP localizes to nucleoli in MCF7(BD) breast epithelial cells (Zhou and Geahlen, 2009). We confirmed their results by expressing C-terminally tagged TRIP-mCherry in HeLa cells and colocalizing TRIP with a nucleolar marker (Fig. 8A). We occasionally observed localization of TRIP-mCherry to nuclear puncta (Fig. 8C).

Due to its low expression levels, hPolη is difficult to detect by immunofluorescence; therefore, its localization is typically assessed in transfected cells overexpressing hPolη (Bienko et al., 2010; Kannouche et al., 2002; Lehmann et al., 2007). To confirm that eGFP-hPolη overexpression does not induce DNA damage, we assessed the presence of γ-H2AX foci, which appear in response to double-stranded breaks. We found that eGFP-hPolη-expressing cells exhibited a decreased (rather than increased) frequency of γ-H2AX-foci (supplementary material Fig. S5); this observation is consistent with a previously described role for hPolη in maintaining genomic stability during unperturbed S phase (Rey et al., 2009).

In cells expressing eGFP-hPolη, hPolη exhibited a diffuse nuclear localization with nucleolar enrichment (Fig. 8B). We occasionally observed hPolη localization to nuclear foci, consistent with its

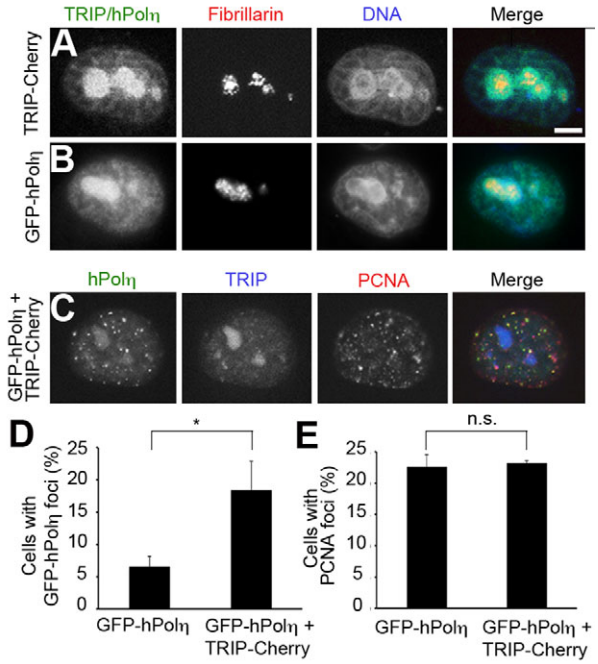

Fig. 8. TRIP promotes recruitment of hPolη to nuclear foci. (A,B) HeLa cells expressing TRIP-mCherry (A) or GFP-hPOLη (B) (green) stained for fibrillarin (red; nucleolar marker) and DNA (blue) display enriched nucleolar localization of both fusion proteins. (C) HeLa cells co-expressing GFP-hPOLη (green) and TRIP-mCherry (blue) display colocalization of GFP-hPOLη with PCNA foci. (D) Increased frequency of GFP-hPOLη foci upon TRIP-mCherry co-expression. Asterisk, P<0.001. (E) Similar frequency of endogenous PCNA foci in cells expressing GFP-hPOLη ± TRIP-mCherry. NS, nonsignificant.

recruitment to stalled replication forks, where it interacts with PCNA during translesion synthesis (data not shown) (Kannouche et al., 2003; Kannouche and Lehmann, 2006). Indeed, we found that most eGFP-hPolη foci colocalized with PCNA foci (supplementary material Fig. S6). When eGFP-hPolη and TRIP-mCherry were co-expressed, a higher percentage of cells had eGFP-hPolη foci, although the average number of eGFP-hPolη foci per cell remained unchanged (Fig. 8C,D; supplementary material Fig. S7). This increased recruitment of eGFP-hPolη to nuclear foci was not secondary to increased formation of PCNA foci (Fig. 8E). Furthermore, the percentage of eGFP-hPolη foci colocalizing with PCNA foci was comparable in cells ± TRIP-mCherry co-expression, consistent with enhanced recruitment of eGFP-hPolη to existing PCNA foci rather than ectopic nuclear foci formation (supplementary material Fig. S6). Taken together, these data suggest that the TRIP/NOPO E3 ubiquitin ligase might regulate Polη by promoting its localization into PCNA foci.

DISCUSSION

We identified members of the Y-family of DNA polymerases as TRIP/NOPO interactors in yeast two-hybrid assays and co-immunoprecipitation experiments. We observed increased sensitivity to UV with *nopo*-like spindles and mitotic arrest of embryos from *dPolη*-null females. We also showed that *dPolη* overexpression in embryos compensates for partial loss of NOPO. We found that TRIP/NOPO enhances Polη ubiquitylation in *Drosophila* embryos and human cells and that TRIP promotes Polη localization to nuclear foci in human cells. Our findings support a model in which NOPO ubiquitylates dPolη during *Drosophila* syncytial embryogenesis to

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promote DNA damage tolerance in order to preserve genomic integrity, proper cell-cycle progression, and continuation of development. One limitation of the current study, however, is that most of the data supporting our model that NOPO acts upstream of dPolη were obtained from assessing the gain-of-function situation. Further studies of loss-of-function mutants of *dPolη* and *nopo* will be required to confirm our findings.

The observation that *dPolη* contributes to DNA damage tolerance in *Drosophila* embryos is consistent with previous studies in *C. elegans* and suggests a conserved role for Y-family DNA polymerases in ensuring cell-cycle progression during development. Germline knockdown of the *dPolη* homolog, *polh-1*, in *C. elegans* caused increased sensitivity of early embryos to UV irradiation, suggesting that POLH-1 is required for genome maintenance during embryogenesis (Ohkumo et al., 2006). POLH-1 has been further proposed to prevent stalling of replication forks during early embryogenesis by replacing the replicative polymerase on chromatin to quickly bypass DNA lesions (Holway et al., 2006; Kim and Michael, 2008).

Our data suggest that NOPO positively regulates *dPolη* activity. *C. elegans* POLH-1 is regulated by sumoylation, which protects it from degradation during lesion bypass (Roerink et al., 2012). POLH-1 ubiquitylation presumably occurs after it has successfully bypassed the lesion and results in ubiquitin-mediated proteolysis by the CRL4-Cdt2 E3 ligase (Kim and Michael, 2008). It will be interesting to determine whether there is a functional homolog of NOPO in *C. elegans* and whether *Drosophila* $CRL4^{Cdt2}$, which regulates endoreduplication cycles by mediating E2F1 degradation, might similarly target *dPolη* for ubiquitin-mediated proteolysis (Zielke et al., 2011). These data would provide evidence for a conserved, highly regulated polymerase-switching model of the replicative and Y-family DNA polymerases during S phase of developing embryos.

We have shown that NOPO enhances Polη ubiquitylation during embryogenesis with reduced levels of this modification in *nopo*-null embryos. We did not observe decreased Polη levels by TRIP overexpression in human cells or in *Drosophila* wild-type versus *nopo*-derived embryos that would indicate TRIP/NOPO-mediated targeting of the polymerase for proteasomal degradation. This observation, along with our genetic interaction and localization data suggesting positive regulation of Polη function, argues against promotion of K48-linked poly-ubiquitylation of Polη by TRIP/NOPO E3 ligase.

We previously showed that NOPO interacts with Bendless (Ben), an E2 ubiquitin-conjugating enzyme that is the *Drosophila* homolog of Ubc13 (Merkle et al., 2009; Muralidhar and Thomas, 1993; Oh et al., 1994; Zhou et al., 2005). In budding yeast, the E2 heterodimer Ubc13-Mms2 mediates K63-linked poly-ubiquitylation of PCNA during postreplicative repair (Hoege et al., 2002). Mammalian Ubc13-Mms2 functions in DNA damage repair and promotion of K63 ubiquitin chain assembly (Andersen et al., 2005). Given that K63-linked ubiquitin chains generally act as nonproteolytic signals modulating protein-protein interactions and are known to facilitate DNA repair, we propose that TRIP/NOPO E3 ligase acts with Ubc13-Mms2 E2 heterodimers to mediate assembly of K63-linked poly-ubiquitin chains on Polη to facilitate bypass of DNA lesions and preserve genomic integrity in human cells and *Drosophila* embryos (Aguilar and Wendland, 2003; Spence et al., 1995).

Studies in budding and fission yeast and mammalian cells have established UV as a major trigger for recruiting Y-family polymerases to mono-ubiquitylated PCNA at sites of stalled replication forks (Lehmann, 2005; Watanabe et al., 2004). In unchallenged cells, mammalian Y-family polymerases localize throughout the nucleus. In response to UV, however, the polymerases are recruited to stalled replication forks and appear as foci on chromatin during S phase (Kannouche et al., 2001; Kannouche et al., 2003; Mukhopadhyay et al., 2004; Murakumo et al., 2006; Ogi et al., 2005). The mechanism by which this controlled intracellular movement of hPolη to sites of DNA damage occurs is not well understood.

The activity of hPolη and its localization to nuclear foci is inhibited by mono-ubiquitylation by the E3 ubiquitin ligase Pirh2 (Bienko et al., 2010; Jung et al., 2011). In response to UV irradiation, Protein kinase C-dependent phosphorylation of hPolη promotes hPolη recruitment to repair foci, and ATR-dependent hPolη phosphorylation contributes to efficient bypass of UV-induced DNA lesions and cell survival (Chen et al., 2008; Göhler et al., 2011). Here we show that co-expression of TRIP and hPolη in cultured human cells similarly promotes increased localization of hPolη to nuclear foci containing PCNA. We propose that TRIP/NOPO-dependent ubiquitylation of Polη results in its translocation to nuclear foci, thereby promoting its recruitment to and interaction with mono-ubiquitylated PCNA on chromatin.

The ubiquitylation state of a protein can be regulated by phosphorylation; this may occur by modulation of E3 ligase activity or facilitation of E3 ligase target recognition (Hunter, 2007). Phosphorylation can induce poly-ubiquitylation that results in proteasomal degradation of target proteins, and studies on components of the TNF-signaling pathway have shown that phosphorylation-dependent K63-linked poly-ubiquitylation can mediate protein-protein interactions or activity of target proteins (Miyamoto et al., 1998; Pickart, 1997). Determining whether TRIP/NOPO-dependent ubiquitylation of Polη and its role in promoting translocation of Polη into nuclear foci require PKC- or ATR-dependent phosphorylation will further enrich our understanding of the mechanisms by which Polη is regulated.

There is evidence that mammalian TRIP, like NOPO, is crucial during development. Mouse embryos lacking TRIP undergo early arrest with proliferation defects and excessive cell death (Park et al., 2007). TRIP also has a reported role in TNF/NF-κB-dependent sexual dimorphism in developing neurons (Krishnan et al., 2009). The authors found an upregulation of TRIP in the developing male anteroventral periventricular (AVPV) nucleus and showed that the size difference in male versus female AVPV nuclei results from apoptosis in developing male neurons (Krishnan et al., 2009). It will be interesting to determine if any of these proposed roles for TRIP in apoptosis and cell proliferation involve its regulation of Y-family DNA polymerases.

Although *dPolη* overexpression suppressed the partial loss of NOPO in embryos from females homozygous for a hypomorphic allele of *nopo*, we did not find obvious developmental defects in *dPolη*-derived embryos. Based on this observation, it is clear that regulation of *dPolη* is not the sole mechanism by which NOPO acts to preserve genomic integrity in early embryos of *Drosophila*. Future work will be necessary to determine whether other Y-family polymerases are involved and to identify additional targets of NOPO E3 ubiquitin ligase that are crucial for maintaining genomic stability and proper progression of development.

MATERIALS AND METHODS

Drosophila stocks

y^1w^{1118} was used as 'wild-type' stock. *UASp-His7-Ub* and mnk^{6006} stocks were gifts from Lynn Cooley (Yale) and Bill Theurkauf (UMass Worcester), respectively. $nopo^{Exc142}$ flies were previously described (Merkle et al., 2009). Other fly stocks were from Bloomington or Szeged stock centers.

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cDNA clones

cDNA clones encoding NOPO, dPolη and dPolι (GH03577, SD05329 and LD29090, respectively) were from the *Drosophila* Gene Collection. Human TRIP cDNA (ID 2821007) was from Open Biosystems. hPolη and hPolκ clones were gifts from Peter Guengerich (Vanderbilt). Supplementary material Table S2 shows primers used for generating DNA constructs.

Transgenesis

For *nopo* rescue experiments, the previously described pCaSpeR4-CG5140 (containing a 3.8-kb genomic fragment including *nopo* and flanking regions) was used as template (Merkle et al., 2009). *nopo* genomic sequence between the 5′ and 3′ UTRs was replaced by *nopo* or human *TRIP* open reading frame (ORF) to generate pCaSpeR4-$nopo_{gen}nopo_{cDNA}$ or pCaSpeR4-$nopo_{gen}TRIP_{cDNA}$, respectively (Fig. 1A). For *dPolη* rescue and overexpression experiments, cDNA was subcloned into modified UASp to generate pUASp-HA-dPolη (Rørth, 1998). Transgenic lines were generated by *P*-element-mediated transformation (Rubin and Spradling, 1982).

Generation of *dPolη* null allele

The *dPolη*-null allele was generated by imprecise excision of *P*-element *EY07711*. $dPol\eta^{Exc2.15}$ contains a 1309 bp deletion in the *dPolη* gene that removes part of the 5′ UTR and exons encoding residues 1-436.

RNA isolation and RT-PCR

RNA STAT-60 (Tel-Test) was used to extract RNA from embryos (0-2 hour). Reverse transcription was performed using the High Capacity cDNA RT Kit (Applied Biosystems) and reaction products used as PCR templates. Supplementary material Table S1 shows RT-PCR primers. PCR products were resolved by agarose gel electrophoresis. For quantitative RT-PCR, RNA was isolated from embryos, third instar larvae, pupae, ovaries and testes of adult flies (2-3 day), and carcasses (minus gonads) of adult flies for cDNA synthesis as described above. Real-time PCR was performed using RT^2 SYBR Green w/Fluorescein qPCR Mastermix (Qiagen) on the Bio-Rad CFX96 Real-Time PCR system. *RP49* was used as internal control. Relative transcript abundance was calculated using the ΔΔCt method. Three independent RNA samples were prepared per genotype. *P*-values were determined using a two-tailed, unpaired Student's *t*-test.

Quantification of hatch rates

Hatch rates (eggs to larvae) were determined as previously described (Merkle et al., 2009). Hatch rate is the ratio of hatched eggs to total eggs laid expressed as a percentage. Two hundred or more embryos were scored per genotype. *P*-values were determined using Fisher's exact test.

Yeast two-hybrid screen

The cDNA library, protocols and reagents were from Clontech. Human *TRIP* cDNA was subcloned into pGBKT7 (bait vector) to encode a fusion protein containing the Gal4 DNA-binding domain. Yeast strain AH109 was transformed with pGBKT7-hTRIP, and fusion protein expression was confirmed by immunoblotting. The bait strain was mated with yeast pre-transformed with a HeLa cDNA library. Transformants with positive interactions were selected on minimal media lacking Leu, Trp and His (triple dropout, TDO). Positive prey plasmids were sequenced and retested on TDO selection medium to confirm interactions.

Yeast two-hybrid assays

Protocols and reagents for yeast two-hybrid assays were from Clontech. cDNAs encoding full-length human TRIP or *Drosophila* NOPO were subcloned into pGBKT7 (bait vector) and transformed into strain AH109. cDNAs encoding full-length or truncated forms of hPolη, hPolκ, dPolη or dPolι were subcloned into pGADT7 (prey vector) and transformed into strain Y187. After mating, diploid cells co-expressing bait and prey constructs were selected by growth on media lacking Leu and Trp (double dropout, DDO). Interactions were tested by spotting cells onto TDO medium (described above) and scoring growth after 3 days at 30°C. Assays were performed three or more times with plating in triplicate.

Cell culture and transfection

HeLa cells were maintained at 37°C and 5% CO_2 in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS), 1% L-glutamine, 100 µg/ml streptomycin, and 100 U/ml penicillin (Gibco). Plasmids made by subcloning cDNAs into tagged pCS2 vectors (2 µg each) were transfected into cells using Lipofectamine 2000 (Invitrogen) for co-immunoprecipitations or Fugene HD (Promega) for immunostaining according to manufacturers' instructions.

Drosophila S2 cells were maintained at 28°C in Schneider's medium containing 10% FBS, 100 µg/ml streptomycin, and 100 U/ml penicillin (Gibco). Plasmids generated by subcloning cDNAs into tagged pRmHa3 vectors (2 µg each) were transfected into cells with calcium phosphate using standard methods. At 24 hours post-transfection, cells were $CuSO_4$-treated (0.5 M) for 24 hours to induce expression.

Co-immunoprecipitation and immunoblotting

HeLa or S2 cells (24 hours post-transfection or post-induction, respectively) were lysed in nondenaturing lysis buffer (NDLB) (50 mM Tris-Cl pH 7.4, 300 mM NaCl, 5 mM EDTA, 1% Triton X-100, protease inhibitors) and centrifuged 10 minutes at 13,000 rpm. For co-immunoprecipitations, lysates (500 µg) were incubated 2 hours with shaking at 4°C with 50 µl of Ultralink Biosupport resin (Thermo Scientific) coupled to anti-MYC (9E10) or anti-FLAG (M2) antibodies. Beads were washed three times with NDLB. Bound proteins and lysates were analyzed by immunoblotting with the following primary antibodies: anti-FLAG (M2, Sigma, 1:1000), anti-c-MYC (9E10, 1:2500), and anti-HA (3F10, 1:500, Roche). Secondary antibodies were conjugated to horseradish peroxidase (HRP). Experiments were performed three or more times with representative results shown.

Drosophila embryos were dechorionated in 50% bleach, washed with water, and lysed in NDLB by homogenization with a pestle. Lysates were centrifuged 10 minutes at 13,000 rpm and analyzed by immunoblotting. Anti-NOPO antibodies were used as previously described (Merkle et al., 2009). Experiments were performed three or more times with representative results shown.

Immunostaining and microscopy

Drosophila embryos (0-2 hours unless otherwise indicated) were collected and dechorionated in 50% bleach (Rothwell and Sullivan, 2000). For UV treatment, dechorionated embryos were exposed to 500 J/m^2 UV (254 nm) in a Stratalinker 1800 (Stratagene) and aged 30 minutes in darkness at 25°C before fixation. Embryos were fixed and devitellinized by shaking in methanol/heptane (1:1) and incubated overnight at 4°C in primary antibodies: anti-α-tubulin (DM1α, 1:500, Sigma), anti-Centrosomin (1:5000, gift from Bill Theurkauf, UMass Worcester), and anti-HA (12CA5, 1:2500). Secondary antibodies were conjugated to Alexa Fluor 488 or Cy5 (1:500, Invitrogen). Embryos were stained with propidium iodide and cleared (Fenger et al., 2000). Mitotic index was determined as previously described (Merkle et al., 2009). Two hundred or more embryos were scored per genotype. *P*-values were determined using Fisher's exact test.

HeLa cells (24 hours post-transfection) were fixed in 4% formaldehyde [20 minutes at room temperature followed by 20 minutes in Tris-buffered saline (TBS) plus 0.05% Triton X-100] or methanol (20 minutes at −20°C followed by washing with TBS plus 0.01% Triton X-100) and blocked in 5% bovine serum albumin (BSA). The following primary antibodies were used: anti-Fibrillarin (38F3, 1:1000, Abcam; formaldehyde fixation), anti-PCNA (clone PC10, 1:1000, Biolegend; methanol fixation), and anti-γ-H2AX (clone JBW301, 1:500, Millipore, Billerica, MA; methanol fixation). Secondary antibodies were conjugated to Cy5 (1:1000, Invitrogen). Cells were mounted in ProLong Gold Antifade Reagent with 4′,6-diamidino-2-phenylindole (DAPI) (Invitrogen). For quantification of GFP-hPOLη and PCNA foci, cells with more than five foci were scored as positive. Experiments were performed three times with ≥200 cells scored per condition. *P*-values were determined using a two-tailed, unpaired Student's *t*-test. Most images were obtained using a Nikon Eclipse 80i microscope with a CoolSNAP ES camera (Photometrics) and Plan-Apo 100×objective. Confocal images used to quantify foci were obtained with a Nikon A1RSi confocal microscope with Plan Apo 60×oil immersion objective.

DNA damage response assays

To test UV sensitivity, embryos (0-2 hour) were dechorionated, irradiated with 0, 250, 500 or 750 J/m^2 UV (254 nm) in a Stratalinker 1800 (Stratagene), and kept in darkness at 25°C before assessing hatch rates. Larval sensitivity to HU was tested as previously described (Rickmyre et al., 2007).

Ubiquitylation assays

For HeLa cell studies, we used an established His_6-ubiquitin method (Campanero and Flemington, 1997; Treier et al., 1994). pMT107 encoding His_6-human ubiquitin was a gift from Bill Tansey (Vanderbilt) (Treier et al., 1994). Twenty-four hours post-transfection, cells were lysed in denaturing buffer (6 M guanidine-HCl, 0.1 M Na_2HPO_4/NaH_2PO_4, 10 mM imidazole) and histidine-tagged proteins purified using Ni-NTA beads (Qiagen) as described (Campanero and Flemington, 1997). Bound proteins and lysates were analyzed by immunoblotting. Experiments were performed three or more times with representative results shown.

For *Drosophila* embryo studies, we used wild-type or *nopo*^{Exc142} females carrying transgenes *UASp-HA-dPolη* plus or minus *UASp-His*$_7$*-Ubiquitin* (gift of Lynn Cooley, Yale) in combination with *nanos-Gal4:VP16* to drive germline expression. Embryo (0-2 hour) lysates were prepared under denaturing conditions, histidine-tagged proteins purified, and bound proteins and lysates analyzed by immunoblotting. Experiments were performed five or more times with representative results shown.

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Competing interests

The authors declare no competing financial interests.

Author contributions

H.A.W., J.A.M. and L.A.L. conceived and designed experiments and analyzed data with intellectual contributions from E.L. and G.B.; experiments were performed by H.A.W. and J.A.M. with assistance from M.C.Y. and T.G.B. in the labs of L.A.L., E.L. and G.B.; H.A.W. and J.A.M. co-wrote the manuscript with guidance from L.A.L.

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Supplementary material

Supplementary material available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.101196/-/DC1

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