REGULATION OF *DROSOPHILA* EARLY EMBRYOGENESIS AND GENOME MAINTENANCE BY THE E3 UBIQUITIN LIGASE NO POLES

By

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To my parents

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

53BP1	p53 binding protein 1
9-1-1	Rad9/Hus1/Rad1
A.g.	*Anopheles gambiae*
AD	activation domain
APC/C	Anaphase-Promoting Complex/Cyclosome
ARF	alternative reading frame
ATM	Ataxia Telangiectasia Mutated
ATP	adenotriphosphate
ATR	ATM and Rad3-related
ATRIP	ATR-interacting protein
AVPV	anteroventral periventricular nucleus
BD	DNA binding domain
BEN	Bendless
BER	base excision repair
BLAST	Basic Local Alignment Search Tool
BRCA1	breast cancer supressor protein 1
bw	*brown*
C	cysteine
C. elegans	*Caenorhabditis elegans*
CAK	Cdk-activation kinase
CBS	cytoskeleton buffer with sucrose

Cdc	cell division cycle
Cdk	Cyclin dependent kinase
Chk1	Checkpoint kinase 1
Chk2	Checkpoint kinase 2
CKI	Cdk kinase inhibitor
cn	*cinnabar*
CREST	calcium-responsive transactivator
CRL4-Cdt2	Cul4-Ddb1-Cdt2
C-term	carboxy terminus
CuSO4	copper sulfate
D.m.	*Drosophila melanogaster*
D.r.	*Danio rerio*
DAPI	4',6-diamidino-2-phenylindole
DDO	double dropout
DDR	DNA damage response
DMEM	Dulbecco's modified Eagle Medium
DNA	Deoxyribonucleic acid
DUB	deubiquitylating enzyme
E	Glutamic acid
E. coli	*Escherichia coli*
E1	ubiqutitin activating enzyme
E2	ubiquitin conjugating enzyme
E3	ubiquitin ligase

EDTA	Ethylene diamine tetraacetic acid
EGTA	ethylene glycol tetraacetic acid
EV	empty vector
FBS	fetal bovine serum
FLAG	FLAG epitope tag
G.g.	*Gallus gallus*
Gal	galactose-induced
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	green fluorescent protein epitope tag
GFS	giant fiber system
grp	*grapes*
GO	Gene Ontology
γTuRC	γ-tubulin ring complex
H	Histidine
h	hour
H.s.	*Homo sapiens*
HA	HA epitope tag
HCl	hydrochloric acid
HECT	Homologous to the E6-AP carboxyl terminus
HeLa	Henrietta Lacks ovarian cell
HLTF	helicase-like transcription factor
HU	hydroxyurea
K	lysine

KCl	potassium chloride
kDa	kilodaltons
LED	light-emitting diode
Leu	leucine
LH	luteinizing hormone
LZAP	LXXLL/leucine zipper-containing alternative reading frame (ARF)-binding protein
M	molar
M phase	mitosis
M.I.	mitotic index
M.m.	*Mus musculus*
mago	*mago nashi*
MAGOH	*mago nashi* homolog
MBP	maltose binding protein
MBT	mid-blastula transition
mCherry	monomeric Cherry epitope tag
mcph1	*microcephalin*
MDM2	murine double minute 2
MEF	mouse embryonic fibroblast
mei	*meiotic*
MES	2-(*N*-morpholino)ethanesulfonic acid
$MgCl_2$	magnesium chloride
mM	milimolar
MMR	mismatch repair

MMS	methyl methanesulfonate
mnk	*maternal nuclear kinase*
MRN	Mre11/Rad50/Nbs1
mRNA	messenger RNA
MT	myc epitope tage
Myc	myc epitope tage
NaCl	sodium chloride
NDLB	non-denaturing lysis buffer
NEB	nuclear envelope breakdown
NEF	nuclear envelope formation
NER	nucleotide excision repair
NF-κB	nuclear factor kappa B
Ni	nickel
nopo	*no poles*
NTA	nitrilotriacetic acid
N-term	amino terminal
ORC	origin recognition complex
P	proline
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PI	propidium iodide

PI3	phosphoinositide 3
PIP	PCNA-interacting peptide
Pirh2	p53-induced protein with a RING-H2 domain
POLH/POLη	DNA polymerase eta
POLI/POLι	DNA polymerase iota
POLK/POLκ	DNA polymerase kappa
pre-RC	pre-replicative complex
Rad	radiation sensitive
Rel	relish
Rev	reversionless
RING	really interesting new gene
RNA	ribonucleic acid
RPA	replication protein A
RT	reverse transcriptase
S	serine
S phase	DNA synthesis
S. cerevisiae	*Saccharomyces cerevisiae*
S. pombe	*Schizosaccharomyces pombe*
SCF	Skp1/Cullin/F-box
SDS	sodium dodecyl sulfate
SHPRH	SNF2 histone linker PHD RING helicase
ssDNA	single-stranded DNA
TBS	Tris buffered saline

TDO	triple dropout
TDT	teral depressor of the trochanter
TGC	trophoblast giant cell
TLS	translesion synthesis
TNF	tumor necrosis factor
TNFR	tumor necrosis factor receptor
TRAF	tumor necrosis factor receptor-associated factor
TRIP	TRAF-interacting protein
Trp	tryptophan
UAS	upstream activating sequence
Ub	ubiquitin
Ubc	ubiquitin conjugating enzyme
UBM	ubiquitin-binding motif
UBZ	ubiquitin-binding ZnF domain
UEV	ubiquitin-conjugating enzyme variant
UTR	untranslated region
UV	ultraviolet
w	*white*
WCL	whole cell lysate
XP-V	Xeroderma pigmentosum variant
Y	tyrosine
y	*yellow*

CHAPTER I

INTRODUCTION

THE CELL DIVISION CYCLE

The process of cell divison involves the exact duplication and equal distribution of the cellular components, including the genetic material, between two daughter cells. A complex network of regulatory components controls the progression through each stage of the cell cycle. Cell-cycle events and machinery are similar among all eukaryotic cells, making it possible to study cell-cycle control in diverse experimental systems. Research from a number of model systems, including the fission yeast *Schizosaccharomyces pombe*, the budding yeast *Saccharomyces cerevisiae*, the fruit fly *Drosophila melanogaster*, and the frog *Xenopus laevis*, has provided tremendous insight into cell-cycle regulation (Thuriaux et al., 1978; Forsburg and Nurse, 1991; Lee and Orr-Weaver, 2003; Philpott and Yew, 2008). In single-cell organisms, cell division results in new daughter organisms. In multi-cellular organisms, cell division is essential for the development of all tissues and organs within the organism. Cell division is a fundamental facet of life, whereas the erroneous segregation of genetic information may result in developmental disorders or disease states, such as cancer.

The genetic material of eukaryotic cells is in the form of DNA, which is packed into discrete DNA-protein structures called chromosomes. Chromosomes and other cellular components must be duplicated once per cell cycle. The canonical cell division cycle involves alternating rounds of DNA replication (DNA synthesis, S phase), in which

the chromosomes are duplicated, and mitosis (M phase), in which the sister chromatids are segregated to the two daughter cells (Fig. 1.1). The canonical cell cycle also consists of intervening gap phases: G1, occurring after mitosis, and G2, occurring after S phase. These two gap phases allow time for cellular growth and serve as important regulatory points to monitor the success of previous cell cycle events. These monitoring stages are called checkpoints, which act as molecular brakes to prevent progression to the next cell cycle stage when spindle or genomic errors are detected.

When mitosis is complete, the new nuclei and organelles must be distributed into separate daughter cells. The resulting daughters should each contain one nucleus, one centrosome, and an equal distribution of cellular organelles. The timing of the separation process, termed cytokinesis, is coordinated with the completion of mitosis. In most eukaryotic cells, a contractile ring, made up of actin bundles and myosin motors, carries out the mechanics of cytokinesis.

CELL-CYCLE REGULATION

Progression through the cell cycle is largely controlled by protein heterodimers of Cyclin dependent kinases (Cdks) and cyclins. The activities of the highly conserved Cdks rise and fall throughout the cell cycle. Association with cyclin proteins via the PSTAIR domain is required for Cdk activity (Morgan, 1996). Cyclins are synthesized and degraded to regulate progression through the cell cycle (Murray and Kirschner, 1989; Glotzer et al., 1991). This oscillatory degradation of cyclins also promotes irreversibility of the cell cycle. Different types of Cdks and cyclins are needed at different cell cycle stages; the resulting Cdk-cyclin combinations control the complexity and periodicity of

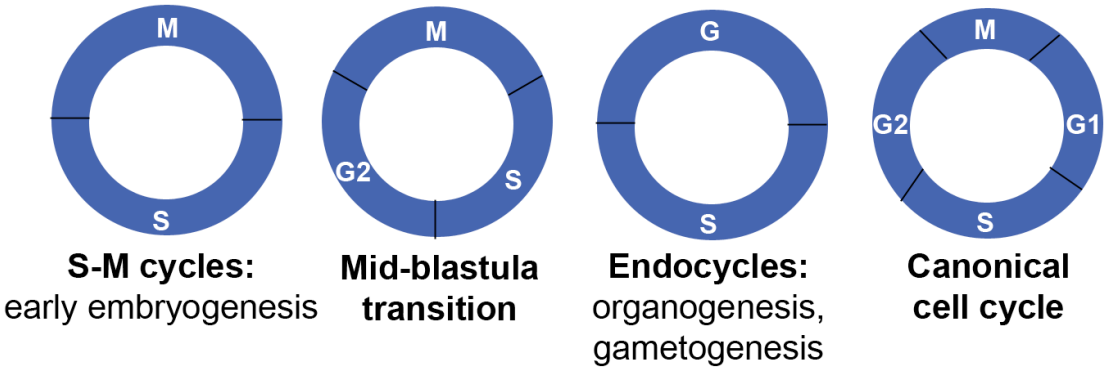


Figure 1.1 Cell division cycles throughout development. Early embryonic cell cycles of *Drosophila*, *Xenopus* and zebrafish consist of rapid, repeating rounds of DNA synthesis (S phase) and mitosis (M phase) with no intervening gap phases. Eventually, a G2 phase is introduced in these organisms at an important developmental switch called the mid-blastula transition (MBT). There also specialized cell cycles during gametogenesis and organogenesis during which cells undergo endoreplication cycles (endocycles); these cycles consist of repeating rounds of S phase and growth (G) without intervening mitoses and result in polyploidy. The canonical cell division cycle involves alternating rounds of S phase and M phase with two intervening gap phases: G1, occurring after M phase of the previous cycle, and G2, occurring after S phase.

the cell cycle. In budding and fission yeasts, a single Cdk is present in association with cell cycle stage-specific cyclins. In mammals, Cdk4/cyclin D and Cdk6/cyclin D are active during G1 to promote cell growth and transition to S phase, respectively. Cdk2/cyclin E promotes entry into S phase, Cdk2/cyclin A is required for completion of S phase, and Cdk1/cyclin A is required for entry into mitosis. Cdk1/cyclin B is required during early mitosis prior to the metaphase to anaphase transition. For the cell to progress through mitosis, cyclin A must be degraded during prometaphase to transition to metaphase and cyclin B must be degraded at metaphase to transition to anaphase (reviewed by Morgan, 1997). This orchestrated modulation of Cdks/cyclins is the engine that drives cell cycle progression.

In addition to requisite cyclin partners, Cdks are subject to additional layers of regulation. Cdks are positively regulated by phosphorylation by the kinase Cdk-activating kinase (CAK), which is a complex of Cdk7/cyclin H. Phosphorylation of Cdk by CAK results in a conformational change to make the Cdk catalytic domain accessible (Lees, 1995). Cdks are also negatively regulated by phosphorylation; Wee1 and Myt1 kinases are responsible for phosphorylating Cdk1 in order to prevent mitotic entry (Gould and Nurse, 1989). These inhibitory phosphorylation events are counteracted by the activity of phosphatases such as Cdc25, which is essential to activate Cdk1/cyclin B for the onset of mitosis (Lew and Kornbluth, 1996). There are also Cdk kinase inhibitors (CKIs) that bind Cdks to keep them inactive (Elledge and Harper, 1994). These mechanisms generate an irreversible feedback loop that controls the activity of Cdks during cell cycle progression. Cyclin protein levels are also highly regulated by the ubiquitin-proteasome system (UPS), which is discussed later in this chapter (Glotzer et al., 1991).

CELL CYCLES THROUGHOUT DEVELOPMENT

There are several "variant" cell cycles that occur during the development of a multicellular organism. These modified cell cycles are specialized, but essential for the development and physiology of many organisms. In some organisms, the early embryo employs a simplified cell cycle consisting of rapid, repeating rounds of DNA replication and mitosis with no intervening gap phases (Fig. 1.1). Such cell cycles are present in the early embryos of *Drosophila melanogaster*, *Xenopus laevis*, and the zebrafish *Danio rerio* (O'Farrell, Stumpff, and Su, 2004; Budirahardja and Gönczy, 2009). The rapid early embryonic cell cycles of these organisms may have evolved for speed due to the exposed environment in which the animals develop. In these early embryonic cycles, cell-cycle regulators in the form of mRNA or protein are provided maternally and slowly depleted. Eventually, a G2 phase is introduced in these organisms at an important developmental switch called the mid-blastula transition (MBT). This also marks the general depletion of the maternal contribution and the onset of transcription of the zygotic genome. As with most cell cycle events, the introduction of a G2 phase and the developmental switch at the MBT are controlled by the activity of Cdks/cyclins (Lee and Orr-Weaver, 2003; Newport and Kirschner, 1984). The regulation of the early embryonic cell cycles of *Drosophila* is discussed later in this chapter.

As development continues, the cell cycles of most organisms become more complex. Typically, the cell cycle after early embryogenesis is canonical in nature, as described earlier in this chapter (Fig. 1.1). There are also examples of variant cell cycles in gametogenesis and organogenesis. For instance, there are several examples of organisms with polyploid cells, which contain more than two copies of the genome. In

Drosophila, the oocyte-supporting nurse cells are polyploid as a consequence of endoreplication cycles (endocycles), which consist of repeated rounds of S phase without intervening mitoses (Lee et al., 2009). Another type of endocycling cell is the placental mouse trophoblast giant cell (TGC), which is crucial for embryo implantation in the uterus (Hoffman and Wooding, 1993). Perturbation of endocycles may result in sterility or organ malfunction.

EARLY *DROSOPHILA* EMBRYOGENESIS

The cell cycles of the early embryo of *Drosophila* are streamlined in nature, thus making it an ideal model system for studying cell-cycle regulation in a multicellular organism. Other major advantages of *Drosophila* as an experimental system include the feasibility of using genetics, cell biology, and biochemistry in examining mutant phenotypes. Early *Drosophila* embryogenesis consists of thirteen rapid S-M (DNA Synthesis-Mitosis) cycles driven by stockpiles of maternally deposited mRNA and protein in a shared cytoplasm (syncytium). These early cell cycles are ~10 minutes in length and consist of oscillating DNA replication and mitosis with no intervening gap phases or cytokinesis (Lee and Orr-Weaver, 2003). Zygotic transcription of most genes does not begin until the fourteenth cycle when cellularization occurs and a G2 phase is introduced at the MBT.

The timing of the early *Drosophila* cell cycles is mostly regulated by the localized accumulation and degradation of cyclins A and B, but the overall pool of cyclin levels remain constant during the early preblastoderm divisions (Edgar et al., 1994). Global cytoplasmic movement and a local oscillation of cyclin concentration are the key factors

in regulating mitosis during early embryonic cell cycles (Ji et al., 2004; Stiffler et al., 1999; Crest et al., 2007). The levels of *Drosophila* Cdc25, String, gradually rise during the first eight syncytial cycles, and then gradually decline; String phosphorylation also fluctuates during these early mitoses (Edgar et al., 1994). Interestingly, however, Cdk1 phosphorylation is not detectable during these cycles, suggesting that inhibitory phosphorylation of Cdk1 does not occur during the syncytial cell cycles of *Drosophila* embryogenesis. As the embryo approaches the MBT and a G2 phase is introduced, the levels of maternal String, Cdk1, and cyclins gradually decline, which allows for the switch to zygotic transcription and more canonical cell-cycle regulation.

THE DNA DAMAGE RESPONSE

In order to monitor the integrity of the genome and to prevent the transmission of genetic errors to daughter cells during cell division, the cell employs regulatory proteins that provide a safeguard mechanism to block cell-cycle progression in the presence of genomic damage. DNA damage can be present at any stage of the cell cycle and exists in many forms, ranging from small single nucleotide alterations to DNA double-strand breaks. DNA damage may also result from many different sources, including spontaneous mutations, chemicals, or radiation. Approximately 30,000 DNA lesions spontaneously occur in a single mammalian cell per day, highlighting the extensive workload presented to the cell to identify and correct lesions (Lindahl and Barnes, 2000). Utilizing a complex network of machinery, the cell will attempt to repair the DNA damage once it has been recognized. The goal of the DNA damage response (DDR) is to prevent transmission of mutated genetic material to progeny (reviewed by (Zhou and

Elledge, 2000). If the DDR is perturbed, this may result in unrepaired DNA damage and uncontrolled proliferation. When mutated DNA is passed to a cell's offspring, the viability of the daughter cells is at stake. In a multicellular organism, this will initially only affect a small population of cells; however, if uncontrolled proliferation is allowed to continue, pathologies such as cancer can result.

MECHANISMS OF DNA DAMAGE DETECTION AND REPAIR

DNA damage must first be detected by proteins monitoring the integrity of the genome at distinct transitions of the cell cycle. In all eukaryotic cells, the DDR is controlled by the Phosphoinositide 3 (PI3)-related kinases: Ataxia Telangiectasia Mutated (ATM) and ATM-related (ATR). These "sensors" associate with damaged DNA, which results in their phosphorylation and activation of the "effector" kinases: Checkpoint kinases 1 and 2 (Chk1 and Chk2). This signaling cascade responds to DNA damage and can activate additional signaling events to repair the damage.

The three possible outcomes of the DDR are DNA repair, cell-cycle arrest or cell death. Some forms of DNA damage can be repaired quickly and do not cause the DDR to trigger a cell-cycle arrest; however, some lesions are more harmful, and a cell-cycle block is initiated. When DNA damage or incomplete DNA replication is detected, the cell activates ATM/ATR to phosphorylate Chk1 and other substrates. Chk1 phosphorylates its downstream targets, such as Cdc25, to initiate a cell-cycle arrest. Cdc25 phosphatases remove the inhibitory phosphates from Cdks, thereby promoting cell-cycle progression. Phosphorylation of Cdc25 in response to checkpoint activation results in its destabilization and cell-cycle delay (Furnari et al., 1997). In other cases, the damage is so

severe or the cell cycle machinery is so perturbed that the cell initiates programmed cell death (apoptosis) (Harper and Elledge, 2007).

In the presence of single-stranded DNA, RPA (replication protein A) recruits ATR and its adapter protein, ATRIP (ATR-interacting protein), which results in the recruitment of the 9-1-1 (Rad9/Hus1/Rad1) complex (Cortez et al., 2001; Ball et al., 2005; Yang and Zou, 2006). Once ATR is activated, it phosphorylates the effector protein Chk1, which can in turn phosphorylate its downstream substrates. In the presence of double-stranded DNA breaks, ATM (and sometimes ATR) is recruited by components of the MRN (Mre11/Rad50/Nbs1) complex, which directs repair of the double-strand break (Lee et al., 2010). In mammalian cells, ATM phosphorylates H2A.X, a variant form of Histone H2A, at sites of double-strand breaks (Burma et al., 2001). There are also several "adaptor" proteins, such as BRCA1 and 53BP1, that are both regulated by and help to regulate the DDR.

In animal cells, but not in budding or fission yeasts, the DDR may result in permanent cell-cycle arrest or cell death. This additional level of protection for the organism is largely controlled by the tumor suppressor p53, the single most frequently mutated protein in cancer cells (Soussi, 2003). p53 is a transcription factor that is activated by ATM and ATR and is required to initiate transcription of downstream target genes involved in preventing cell-cycle progression and triggering apoptosis (Hirao et al., 2000). A major target of p53 is the Cdk inhibitor p21, which inhibits Cdk/cyclin and prevents cell-cycle progression at G1/S (Shivji et al., 1994). If the DDR is unable to repair the damage in a timely manner, cells may initiate p53-mediated apoptosis.

THE DNA CHECKPOINT IN THE EARLY *DROSOPHILA* EMBRYO

The syncytial *Drosophila* embryo lacks a canonical G2 phase (Fig. 1.1). During early *Drosophila* embryogenesis, *mei-41*, the ATR homolog, and *grapes* (*grp*), the Chk1 homolog, are required to slow the late syncytial cell cycles (11-13) in order to introduce a G2 phase at cellularization (Sibon et al., 1999; Fogarty et al., 1994; Sibon, Stevenson, and Theurkauf, 1997). These checkpoint kinases are not activated in the early embryo in response to DNA damage or incomplete replication until late syncytial divisions. *mei-41*/*grp* promote Cdk1 inhibitory phosphorylation to allow the switch to zygotic control at the MBT after cycle 13. According to a well-accepted model, critical replication factors are depleted near the end of syncytial embryogenesis, and mitotic entry is likely slowed in these cell cycles to allow time to complete replication (Sibon, Stevenson, and Theurkauf, 1997; Crest et al., 2007; McCleland, Shermoen, and O'Farrell, 2009; Lu et al., 2009). Embryos from *mei-41* or *grp* mutant females fail to lengthen interphase in these cycles and enter mitosis with incompletely replicated DNA.

CHECKPOINT KINASE 2-MEDIATED CENTROSOMAL INACTIVATION IN THE EARLY *DROSOPHILA* EMBRYO

When DNA damage or incomplete DNA replication occurs in the early *Drosophila* embryo, as in the *mei-41* or *grp*-derived embryos, Checkpoint kinase 2 (Chk2) is activated in the vicinity of affected nuclei. This causes a cell-cycle arrest with centrosomal, spindle, and chromosomal defects (Takada et al., 2007). This Chk2-dependent phenomenon, referred to as centrosomal inactivation, occurs as a result of dissociation of γ-tubulin ring complex (γTuRC) proteins from core centrosomal subunits

(Sibon et al., 2000; Takada et al., 2003). Defective Chk2-activated nuclei drop into the interior of the embryo in later syncytial cycles and are not cellularized at the MBT, so as to be excluded from the embryo proper. This culling of Chk2-activated nuclei with damaged or incompletely replicated DNA protects the genomic integrity of the developing *Drosophila* embryo. In mammalian cells, Chk2 is localized to the centrosome and inhibition of centrosome separation has been observed in response to DNA damage (Fletcher et al., 2004; Fletcher and Muschel, 2006). Whether or not a similar role for Chk2 in centrosomal inactivation occurs in mammalian cells remains controversial (Song, 2005).

CELL CYCLE MISREGULATION AND TUMORIGENESIS

The importance of the DDR is evident in the human diseases caused when it is missing or dysfunctional. Spontaneous mutations arise with each cell division, and if those mutations are not corrected or if the mutated cells are not prevented from dividing, DNA damage will accumulate. This may eventually result in misregulated cell growth and division and ultimately increase the likelihood of cancer. Cancer is uncontrolled cell growth, which can result from the mutation or dysfunction of key cell-cycle regulators. When cancer cells invade neighboring cells and tissue, this is termed metastasis. A predisposition to cancer may be inherited genetically, when a mutation or multiple mutations are transmitted from parent to progeny. Mutations resulting in cancer may also occur spontaneously or as a consequence of the environment. Chemicals and radiation from the environment can be toxic to the genome and cause a range of damage to the DNA, from single nucleotide alterations to breaks in the double helix. Cancer has

recently been the leading cause of death in economically developed countries and the second leading cause of death in developing countries (Jemal et al., 2011). A more complete understanding of the cell-cycle machinery and how cancers progress will potentially lead to more effective therapeutics in order to fight these diseases.

Cell-cycle dysfunction during the development of multicellular organisms is also a potential cause of pathogenesis. Cell-cycle components must be properly functioning and regulated during critical developmental stages; if their regulation is perturbed, sterility or developmental disorders may result. For example, the exact timing of the degradation of CDC25-1 during embryogenesis of the nematode *Caenorhabditis elegans* is needed to establish the development of the gut and the germline (Bao et al., 2008). Without the establishment of these crucial cell lineages, the survivability of the developing organism is at stake.

PROTEIN UBIQUITYLATION

A posttranslational modification of proteins that involves the covalent attachment of ubiquitin was discovered by Aaron Ciechanover, Avram Hershko and Irwin Rose in the early 1980s (Hershko et al., 1980, 1983; Hershko and Ciechanover, 1998). Ubiquitylation is a highly dynamic, multi-step process that involves four key components: a ubiquitin activating enzyme (E1), ubiquitin conjugating enzymes (E2s or Ubcs), ubiquitin ligases (E3s), and ubiquitin (Ub) (reviewed by Fang and Weissman, 2004; Weissman, 2001; Wilkinson, 2000) (Fig. 1.2). Ubiquitin is a small (76 amino acids) protein that is highly conserved and universally distributed among all eukaryotic cells. Ubiquitylation can involve the linkage of one or more Ub molecules to another

protein. Protein ubiquitylation may alter its fate in a number of ways, including the following: targeting it for destruction by the 26S proteasome, changing its subcellular location, or changing its protein-protein interactions (Glickman and Ciechanover, 2002). Protein ubiquitylation affects a variety of cellular processes, such as protein processing, cell cycle control, chromatin remodeling, DNA repair and membrane trafficking (Liu and Chen, 2011; Broemer and Pascal Meier, 2009; Le Bras et al., 2011; Acconcia et al., 2009; Hershko, 1997; Al-Hakim et al., 2010; O'Connell and Harper, 2007).

MECHANISM OF PROTEIN UBIQUITYLATION

The ubiquitylation process begins when an E1 enzyme activates Ub at its carboxy terminus to form a thiolester bond between E1 and Ub in an ATP-dependent mechanism (Fang and Weissman, 2004). Ub is then transferred by an E2 enzyme, in association with an E3 ligase, to its substrate protein. Ub attaches to substrates via isopeptide bonds at key lysine residues. In the final step of the "canonical" ubiquitin-proteasome pathway, the 26S proteasome recognizes ubiquitylated proteins, which are degraded into smaller peptides in an ATP-dependent manner. To begin a new cycle, Ub molecules are recycled by deubiquitylating enzymes (DUBs) (Komander et al., 2009). In *S. cerevisiae*, there is one E1, 11 E2s, and >50 E3s (Chan and Hill, 2001). In *Drosophila*, there is one E1, ~40 E2s, and ~130 E3s as predicted by database searches. *Drosophila* has 61% fewer components of the ubiquitylation machinery than humans, making it an attractive model organism for studying protein ubiquitylation in a multicellular context (Ditzel and Meier, 2005; Bergmann, 2010).

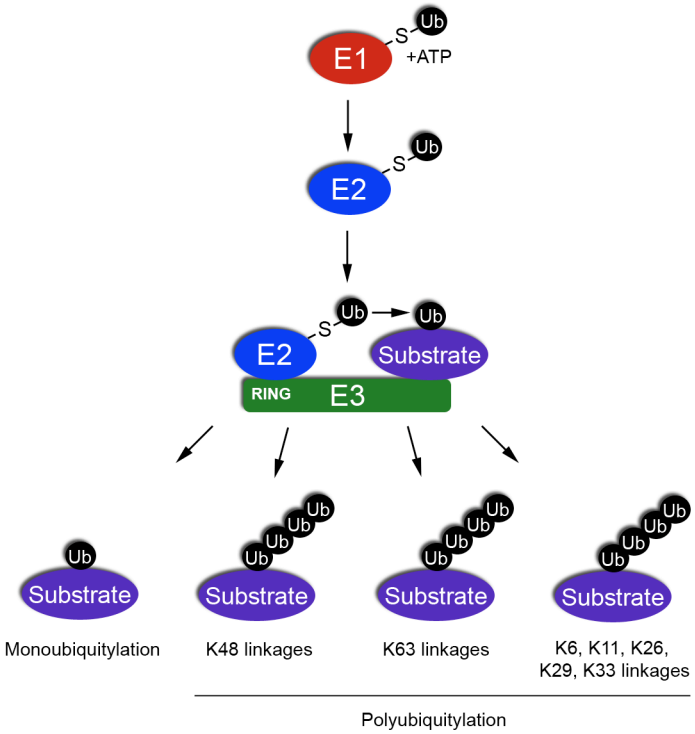


Figure 1.2 Mechanisms of protein ubiquitylation. Modification of proteins by ubiquitylation is a multi-step process that involves four key components: an ubiquitin activating enzyme (E1), an ubiquitin conjugating enzymes (E2), an ubiquitin ligase (E3), and ubiquitin (Ub). E1 enzyme activates Ub in an ATP-dependent mechanism to form a thiolester bond between E1 and Ub. Ub is then transferred to an E2 enzyme. The E3 associates with an E2 via its RING domain, as well as with its substrate protein. The E2 transfers Ub to the substrate via isopeptide bonds at key lysine residues. The attachment of Ub to its substrate may involve a single Ub molecule (monoubiquitylation) or multiple Ub molecules to each other to form an Ub chain (polyubiquitylation). In an Ub chain, Ub molecules may be linked together via any of Ub's seven lysine (K) residues, resulting in K48, K63, K11, K29, K33, K6, or K27 linkages.

Not all ubiquitylated proteins are targeted to the 26S proteasome for degradation. Determining factors that regulate the fate of a substrate include the site(s) of modification and the structure of the ubiquitin chain (if polyubiquitylated). The attachment of Ub to its substrate may involve a single Ub molecule (monoubiquitylation), multiple Ub molecules to several sites on the same substrate protein (multiubiquitylation), or multiple Ub molecules to each other to form a Ub chain (polyubiquitylation). Monoubiquitylation has been implicated in a number of cellular processes, including DNA repair and membrane trafficking (Al-Hakim et al., 2010; Haglund et al., 2003; Lee and Myung, 2008). The occurrence of poly-Ub chains *in vivo* at each of Ub's seven lysine residues has been reported (Xu et al., 2009). When Ub forms a chain, individual Ub molecules may be linked together via lysine 48 (K48), lysine 63 (K63), lysine 11 (K11), lysine 29 (K29), lysine 33 (K33), lysine 6 (K6), or lysine 27 (K27) of Ub (Fig. 1.2) (Johnson et al., 1995; Hatakeyama et al., 2001; Kirkpatrick et al., 2006; Nishikawa et al., 2004). Although the K48-linkage typically serves as a degradation signal and is the most widely studied Ub linkage, other Ub sites have been shown to be involved in important cellular processes. For example, Ub chains linked via K63 are important in DNA repair, NF-κB activation and endocytosis (Panier and Durocher, 2009; Iwai and Tokunaga, 2009; Lauwers et al., 2010). K63-linkages often act as non-proteolytic signals, suggesting that Ub chains serve as distinct signals within the cell, resulting in specific and varied downstream cellular events.

E3 UBIQUITIN LIGASES

E3 ubiquitin ligases serve as the specificity factors during ubiquitylation, as they bind both the E2 conjugating enzyme (E2) and the substrate to be ubiquitylated. There are two main classes of E3s: HECT (Homologous to the E6-AP Carboxyl Terminus) domain-containing E3s and RING (Really Interesting New Gene) domain-containing E3s (Bernassola et al., 2008; Deshaies and Joazeiro, 2009). The RING-type E3 ubiquitin ligase contains a specialized zinc finger motif consisting of 40 to 60 residues that binds two atoms of zinc. This domain is likely involved in mediating protein-protein interactions, most commonly to its partner E2(s) (Joazeiro and Weissman, 2000). The RING domain structure is referred to as a 'cross-brace' motif because of the spacing of zinc ions within the motif. There are two different classes of RING domains that differ in their cysteine/histidine pattern: the C3HC4-type and the C3H2C3-type, which is also referred to as the RING-H2-type (Ardley and Robinson, 2005).

PROTEIN UBIQUITYLATION AND DEGRADATION DURING THE CELL CYCLE

During the cell cycle, ubiquitylation temporally controls the degradation of many proteins, including the mitotic cyclins. Two multi-subunit E3s involved in promoting the ubiquitin-mediated proteolysis of cell cycle components are the Anaphase-Promoting Complex/Cyclosome (APC/C) and the SCF (Skp1/Cullin/F-box) complex. The SCF complex consists of three core subunits, the RING protein Rbx1, the Cullin Cul1, and Skp1. The substrate binds to a substrate-specific F-box protein that is also bound to the Skp1 subunit of the SCF. The SCF complex is responsible for ubiquitylating and

degrading G1/S cyclins, Cdk1 inhibitors, and other cell cycle components (Skaar and Pagano, 2009).

The APC/C consists of twelve subunits, including a RING-containing subunit APC11, and is active at the metaphase to anaphase transition through G1 (Simpson-Lavy et al., 2010; Manchado et al., 2010). The APC/C must first be activated by one of its effector proteins, Cdc20 or Cdh1. Cdc20 (Fizzy in *Drosophila*) activates the APC/C at the metaphase to anaphase transition and is responsible for the ubiquitylation and destruction of Securin, which allows for the release of Separase. Once free, Separase destroys sister-chromatid cohesion and allow for the progression to anaphase. Mitotic cyclins are also degraded by the APC/C at metaphase to inactivate Cdk1. Cdh1 (Fizzy-related in *Drosophila*) activates the APC/C in late mitosis to ensure that S phase and mitotic cyclin levels are kept low and Cdk1 activity is inhibited. At the end of G1, the APC itself is targeted for degradation in order for cyclin levels to rise and initiate S phase.

DNA REPLICATION AND THE IMPORTANCE OF PCNA

Duplication of the genome occurs during S phase, and the accurate transmission of the genetic material to the daughter cells is necessary to maintain genomic integrity. DNA replication is the process by which the genome is duplicated, and normally this occurs rapidly and with high accuracy in eukaryotic cells (2900 bases per minute) (Moldovan et al., 2007). Beginning at regions of the DNA called origins of replication, DNA replication is initiated when a pre-replicative complex (pre-RC) is formed during G1, thereby licensing the cell to begin S phase. The formation of the pre-RC is catalyzed

by the binding of the proteins Cdc6 and Cdt1 to the six-subunit ATPase called the origin recognition complex (ORC) at sites of replication (replication foci) (Prasanth et al., 2004). Once an origin is activated, the pre-RC disassembles and cannot reassemble until the next G1, allowing only one round of duplication of the genome per cell cycle. DNA replication proceeds when Polα, the priming DNA polymerase, binds the DNA synthesis machinery at the replication fork and begins to synthesize new DNA strands. The replicative polymerases, Polδ and Polε, continue DNA replication until the entire chromosome is duplicated.

One of the key players in both DNA synthesis and DNA repair is the homotrimeric DNA clamp Proliferating cell nuclear antigen (PCNA). This sliding clamp protein is structurally and functionally conserved in all eukaryotes. PCNA forms a ring-like structure that encircles the DNA during replication and serves as a linker between DNA and the replicative DNA polymerase (Polδ or Polε). PCNA is considered a processivity factor during DNA replication; studies in budding yeast have shown that its presence increases the progression and accuracy of DNA polymerases 100-fold (Arroyo et al., 1996). PCNA is also involved in many DNA repair processes, including the prevention of sister-chromatid recombination, mismatch repair (MMR), base excision repair (BER), and nucleotide excision repair (NER) (reviewed by Moldovan et al., 2007).

PCNA REGULATION DURING DNA REPLICATION AND REPAIR

PCNA activity is highly regulated by posttranslational modifications. PCNA undergoes monoubiquitylation, polyubiquitylation, and sumoylation in response to different cellular signals (reviewed by Shaheen et al., 2010; Lee and Myung, 2008;

Moldovan et al., 2007). While some of the enzymes responsible for modifications on PCNA have been uncovered, the signaling events that lead to these events are not well understood. Furthermore, while many of the players in PCNA regulation have been reported in budding and fission yeasts, much less is known about PCNA regulation in higher organisms.

In *S. cerevisiae*, PCNA is monoubiquitylated by the E3 ubiquitin ligase Rad18 in conjunction with the E2 conjugating enzyme Rad6 in response to a stalled replication fork (Bailly et al., 1997). Monoubiquitylation of PCNA at lysine 164 (K164) in turn recruits a specialized DNA polymerase needed to bypass the damaged DNA in a process called translesion synthesis (TLS), which is discussed later in this chapter (Haracska et al., 2006). PCNA is also sumoylated at K164 in order to prevent recombination and sister-chromatid cohesion during S phase (Watts, 2006). When the DNA damage is so severe that TLS cannot bypass the lesion at the stalled replication fork, a K63-linked polyubiquitin chain is elongated from the monoubiquitin on PCNA by another E2/E3 complex, MMS2-Ubc13/Rad5 (Broomfield et al., 1998; Torres-Ramos et al., 2002; Haracska et al., 2006). This polyubiquitylation of PCNA inhibits TLS and initiates an error-free pathway of DNA repair; replication is formally stalled until the lesion is successfully corrected.

In mammalian cells, PCNA regulation is more complex, and the components involved in these events are less well understood. Similar to budding and fission yeast, PCNA is monoubiquitylated by the E2/E3 pair Rad6B/Rad18 in order to initiate TLS (Hoege et al., 2002). Although Rad18 is essential for TLS activation in mammalian cells, Rad18-independent monoubiquitylation of PCNA has been reported (Simpson et al.,

2006). PCNA has also been shown to be polyubiquitylated in mammalian cells, although identification of the E2(s)/E3(s) responsible for this modification is still uncertain. The mammalian E3 ligase Rad5 homologs are HLTF (helicase-like transcription factor) and SHPRH (SNF2 histone linker PHD RING helicase), and polyubiquitylation of PCNA by these E3 ligases has been shown in cells overexpressing these proteins (Unk et al., 2006; Motegi et al., 2008; Lin et al., 2011; Krijger et al., 2011). These studies reported HLTF and SHPRH independently catalyze a K63-linked polyubiquitin chain on PCNA, and this modification prevents TLS without promoting the degradation of PCNA. Double knockout mice lacking these E3s are viable, however, and PCNA polyubiquitylation occurs normally in MEFs (mouse embryonic fibroblasts) derived from the doubly mutant embryos (Krijger et al., 2011). These data suggest that other E3 ligases are likely important for PCNA regulation in higher organisms, either in concert with, or independent of, the Rad5 homologs HLTF and SHPRH.

It is worth noting that PCNA was originally considered a "cyclin," as its protein levels oscillate during the cell cycle in higher eukaryotes (Moldovan et al., 2007). As suggested by its name, PCNA levels are high in dividing cells, but levels peak in S phase. PCNA levels are thought to be regulated by the ubiquitin-proteasome system throughout the cell cycle, although the enzymes and resulting modifications responsible for its degradation have not yet been reported.

TRANSLESION DNA SYNTHESIS

As discussed previously, the threat of DNA damage can be addressed using a number of cellular responses. The eukaryotic cell possesses many mechanisms to repair

DNA damage, such as homologous end-joining, non-homologous end-joining (NHEJ), homologous recombination, single-strand annealing, NER, BER, and MMR. Cells also possess mechanisms to temporarily tolerate DNA damage until the DNA repair machinery can effectively correct the problems. These "tolerant" processes, which include two parallel pathways, are triggered during S phase when the replication machinery senses damage (Waters et al., 2009). Translesion synthesis (TLS) is one measure taken to bypass damaged DNA during S phase (Fig. 1.3). This process is mediated by PCNA and its recruitment of non-canonical DNA polymerases to sites of damage (reviewed by Shaheen et al., 2010; Lehmann, 2006). These specialized polymerases include Polζ (also referred to as DNA polymerase zeta or Rev3), a member of the B family of DNA polymerases, and members of the Y family of DNA polymerases; the latter, consisting of Rev1, Polι, Polη, and Polκ, are discussed in the next section (Waters et al., 2009). Although the genes encoding the TLS polymerases have been known for about 40 years, it is only in the past decade that the process of TLS and the associated DNA polymerases has been studied in detail (Lemontt, 1971; Waters et al., 2009).

TLS promotes the completion of DNA replication, instead of repairing damaged DNA. The process is initiated by the monoubiquitylation of PCNA on K164 by Rad6/Rad18, which subsequently triggers the recruitment of translesion DNA polymerases to the replication fork (Friedberg et al., 2002). The TLS polymerases use the damaged DNA as a template to incorporate a nucleotide opposite to the lesion. Sometimes this incorporation occurs with high accuracy; at other times, however, the

bypass event is mutagenic and must be repaired later. The mechanism of TLS by the Y polymerases is discussed in more detail below.

THE Y FAMILY OF DNA POLYMERASES

DNA polymerases are enzymes that incorporate deoxynucleotides into DNA during replication or repair. DNA polymerases play an essential role in duplicating the genome and preserving genomic integrity. The Y family of DNA polymerases is composed of non-canonical DNA polymerases that facilitate TLS. These specialized polymerases have been shown to interact with monoubiquitylated PCNA at the replication fork in response to the detection of DNA lesions (Andersen et al., 2008). From studies in bacteria, yeasts and mammalian cells, ultraviolet (UV) light has been shown to be a major trigger to recruit Y-family polymerases to monoubiquitylated PCNA at sites of stalled replication forks (Waters et al., 2009). In unchallenged cells, Y-family polymerases are localized diffusely throughout the nucleus during interphase. In the presence of UV, however, the polymerases appear as nuclear foci during S phase (Andersen et al., 2011).

The Y family of DNA polymerases is conserved from bacteria to humans (Waters et al., 2009; Jarosz et al., 2007; Lehmann, 2006). In mammals, there are four Y-family DNA polymerases: Polκ (POLK), Polι (POLI), Polη (POLH) and REV1. The members of the Y family of polymerases have several conserved protein domains that allow them to interact with their associated binding partners and to effectively mediate TLS. All four mammalian polymerases have a catalytic DNA polymerase domain at their amino termini (Parker et al., 2007; Prakash et al., 2005; Lehmann, 2006). They also all have ubiquitin

binding domains, either in the form of UBMs (ubiquitin-binding motifs) or UBZs (ubiquitin-binding ZnF domains). The Y polymerases can interact with each other. POLH, POLI and POLK can all interact with REV1 via defined REV1-interacting domains; REV1, in turn, has a defined region at its carboxy terminus that is responsible for its interaction with the other Y polymerases (Ross et al., 2005; D'Souza and Walker, 2006). POLH, POLI and POLK have PCNA interacting domains called PIP (PCNA-interacting peptide) boxes. Interestingly, POLH, POLI and POLK interact directly with PCNA via a PIP box and indirectly via an ubiquitin-binding domain. These domains are necessary for the recruitment and interaction of Y-family DNA polymerases with monoubiquitylated PCNA and are also essential for their function in TLS (Haracska et al., 2006; Acharya et al., 2008; Bienko et al., 2005; Andersen et al., 2008).

Mutation of one of the human Y-family DNA polymerases, POLH, results in Xeroderma Pigmentosum Variant (XP-V), a disease characterized by UV sensitivity with increased incidence of skin cancer (Masutani et al., 1999; Kannouche and Stary, 2003; Kannouche et al., 2001). A mouse model of XP-V developed by generating *polh*$^{-/-}$ null mutants exhibit high mutation frequencies and UV-induced epithelial tumors (Busuttil et al., 2008; Ohkumo, Kondo, et al., 2006). The high frequency of mutations in individuals with XP-V supports the model that POLH is needed to bypass lesions during TLS and also suggests that other DNA polymerases that compensate for a POLH-deficiency are highly inaccurate at bypassing UV-induced lesions. These observations challenge the preconceived idea that TLS is an error-prone pathway in response to UV.

The *Drosophila* genome encodes three members of the Y family of DNA polymerases: DNApol-eta, DNApol-iota and Rev1. Little has been reported on

Drosophila Y polymerases, and there are currently no reported mutant alleles for these genes in flies. As in other organisms, recombinant *Drosophila* DNApol-eta and DNApol-iota proteins function as translesion polymerases *in vitro* (Ishikawa et al., 2001). Domain mapping of the protein-protein interactions between fly Y polymerases has also been reported. These data show that DNApol-eta and DNApol-iota both interact with Rev1 via yeast two-hybrid assays (Kosarek et al., 2008). Interactions of the Y-family polymerases with each other are consistent with reports for the Y polymerases conserved in budding and fission yeast, *C. elegans* and mammals.

MODELS FOR LESION BYPASS BY TLS POLYMERASES

The polymerase-switching model proposes a mechanism of lesion bypass at the replication fork (Kannouche et al., 2004; Friedberg et al., 2005; Lehmann et al., 2007; Kannouche and Lehmann, 2004; Andersen et al., 2011). TLS polymerases are first recruited and activated by their interaction with monoubiquitylated PCNA at the replication fork in the presence of damaged DNA (Fig. 1.3). The first switch occurs when the TLS polymerase takes the place of the replicative polymerase, thus allowing the TLS polymerase to incorporate a nucleotide across from the lesion. The second switch occurs once the lesion is bypassed: the replicative polymerase comes back on to the chromatin, and DNA synthesis resumes.

The mechanisms by which the "switches" occur are not well understood. One way that Y polymerases may be regulated is by ubiquitylation. In budding yeast, levels of the POLH homolog Rad30 fluctuate in response to UV treatment. Although the ubiquitin machinery involved has not yet been identified, data suggest that Rad30 undergoes

ubiquitin-mediated proteolysis when it is not needed (i.e. in the absence of DNA damage) and is stabilized after UV treatment (Skoneczna et al., 2007). In mammalian cells, levels of POLH have also been shown to change in response to cellular stress. The E3 ligase Pirh2 may play a role in regulating POLH protein levels, although promotion of ubiquitin-mediated proteolysis by this E3 ligase has not been shown (Jung et al., 2010). Mammalian POLH has also been shown to be monoubiquitylated in the absence of damaged DNA (Bienko et al., 2010). As with other reports on Y polymerase regulation, the machinery that regulates this modification has not been reported, and the authors of this work even suggest that the monoubiquitylation of POLH may be E3-independent. In all, it appears from the limited studies on the regulation of Y polymerases that they are posttranslationally modified, likely by an ubiquitylation event, to hold them inactive in the absence of DNA lesions.

Although there is growing data in support of the polymerase-switching model, it is restricted to times of active DNA replication. The second model, the gap-filling model, has been proposed to account for the roles of TLS polymerases outside of DNA replication. In this model, TLS polymerases are recruited to stretches of single-stranded DNA (ssDNA) gaps and subsequently fill in the gaps by synthesizing across the lesion using the opposite DNA strand as a template (Waters et al., 2009). Evidence in support of the gap-filling model is currently restricted to *S. cerevisiae*, although it is possible that both polymerase switching and gap filling occur in all organisms depending on the lesion and the cell-cycle stage.

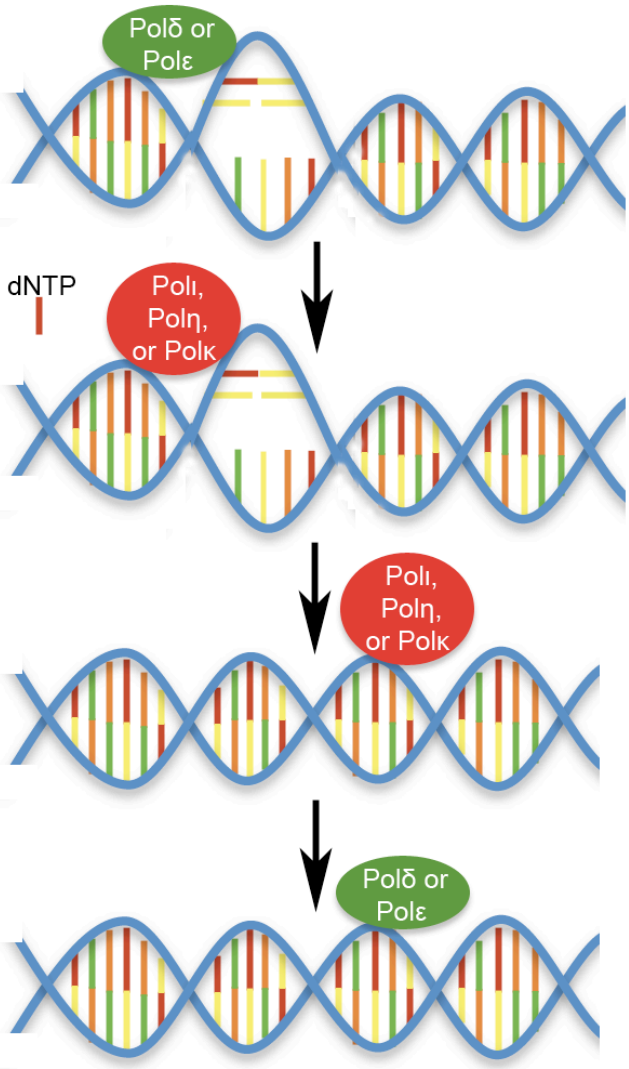


Figure 1.3. Translesion synthesis (TLS). TLS is defined by the replicative bypass of DNA damage. This process is mediated by PCNA and its recruitment of members of the Y family of DNA polymerases, consisting of Rev1, Polι, Polη and Polκ. One model proposed to explain the mechanism of lesion bypass at the replication fork is polymerase-switching. The first switch occurs when the TLS polymerase takes the place of the replicative polymerase (Polδ or Polε), thus allowing the TLS polymerase to incorporate a nucleotide across from the lesion. The second switch occurs once the lesion is bypassed; the replicative polymerase is recruited back to the chromatin, and normal DNA synthesis resumes.

THE ROLE OF Y-FAMILY DNA POLYMERASES IN *CAENORHABDITIS ELEGANS* EMBRYOGENESIS

Although little has been reported on potential roles for the Y-family polymerases or TLS during development, mRNA expression data sets show that Y-family polymerases are highly expressed during gametogenesis and embryogenesis of many developing organisms. Similar to *Drosophila*, early embryonic cell cycles of *C. elegans* are rapid and maternally controlled (Bao et al., 2008). Knockdown of *polh-1*, the *C. elegans DNApol-eta* homolog, in the female germ line results in increased sensitivity of early embryos to UV radiation (Ohkumo et al., 2006). This finding suggests that POLH-1, and perhaps TLS, plays a critical role during early embryogenesis in worms. POLH-1 has been further proposed to prevent stalling of replication forks during the early embryonic cell cycles of *C. elegans* by quickly responding to and bypassing lesions (Holway et al., 2006; Kim and Michael, 2008). The authors hypothesize that POLH-1 takes the place of replicative polymerases during S phase (via a polymerase-switching event) at this developmental stage, thereby keeping the rapid early embryonic cell cycles progressing on schedule.

C. elegans POLH-1 has been shown to be modified by sumoylation via the SUMO E3 GEI-17 and by ubiquitylation via the Cul4-Ddb1-Cdt2 (CRL4-Cdt2) ubiquitin ligase (Kim and Michael, 2008). While these results suggest that sumoylation positively regulates POLH-1 by protecting it from degradation, modification of POLH-1 by ubiquitylation presumably occurs once POLH-1 has successfully bypassed the lesion to prevent the polymerases from binding to chromatin. Although ubiquitylation or ubiquitin-mediated proteolysis by CRL4-Cdt2 has not specifically been shown, levels of POLH-1 are stabilized when *cdt-2* is knocked down in the *C. elegans* embryo.

In conclusion, there appears to be a unique role for the Y-family DNA polymerases during early embryonic cell cycles. Precise regulation of POLH-1 during *C. elegans* embryogenesis may be important to keep the rapid cycles on schedule, as well as to keep it away from the chromatin when it is not needed. These data support a highly regulated polymerase-switching model of the replicative and Y-family DNA polymerases during S phase of developing embryos. Given that expression of the Y polymerases during early embryogenesis is highly conserved in eukaryotes, it seems likely that these polymerases play important roles during this developmental window in other organisms.

In the following chapters, I will present my work analyzing the roles of NOPO (No Poles) in the regulation of *Drosophila* early embryogenesis and genome maintenance. I will also present my work investigating interactors of NOPO's mammalian homolog, TRIP (TRAF-interacting protein). My identification and characterization of NOPO and TRIP as E3 ubiquitin ligases should provide critical insight into the mechanisms underlying cell-cycle progression and genome maintenance.

CHAPTER II

no poles ENCODES A PREDICTED E3 UBIQUITIN LIGASE REQUIRED FOR EARLY EMBRYONIC DEVELOPMENT OF *DROSOPHILA*

The contents of this chapter have been published (Merkle et al. 2009).

INTRODUCTION

To ensure faithful transmission of the genome upon cell division, eukaryotic cells have developed checkpoints, regulatory pathways that delay cell-cycle progression until completion of prior events. The DNA damage/replication checkpoint plays a critical role in preserving genomic integrity (Branzei and Foiani, 2008). Upon detection of DNA defects, the kinases ATM (ataxia telangiectasia mutated) and ATR (ATM-Rad3-related) are recruited to sites of damage and activated. ATM and ATR substrates include checkpoint kinases Chk1 and Chk2, which phosphorylate proteins that mediate cell-cycle arrest. The ensuing delay, resulting from engagement of this checkpoint, presumably allows cells time to correct defects.

Research over the past decade has highlighted major roles for protein ubiquitination in regulating cellular responses to DNA damage (Harper and Elledge, 2007). This post-translational modification, which involves covalent linkage of one or more ubiquitin molecules to another protein, regulates many fundamental cellular processes (Pickart, 2001). Ubiquitination may alter a protein's fate in numerous ways,

such as targeting it for destruction by the 26S proteasome, changing its subcellular location, or changing its protein-protein interactions.

Ubiquitination is a highly dynamic, multi-step process that requires three components: ubiquitin activating enzyme (E1), ubiquitin conjugating enzyme (E2 or Ubc), and ubiquitin ligase (E3). E3s can be divided into two main classes: HECT and RING domain-containing proteins. RING-type E3 ubiquitin ligases (Freemont, 2000; Jackson et al., 2000) contain a specialized motif of 40 to 60 residues that binds two zinc atoms. Many RING-type E3s bind to partnering E2 conjugating enzymes via their RING domains (Passmore and Barford, 2004). Database searches of the *Drosophila* genome predict that it contains one E1, 36 E2s, and ~130 E3s, which represents ~40% of the ubiquitination machinery in humans (Ditzel and Meier, 2005).

Significant insights into the roles of many cell-cycle regulators have come from studying their functions in *Drosophila*. *Drosophila* is well suited for studying cell-cycle regulation during formation of a multicellular organism, in large part due to its developmental use of cell cycles differing in structure from canonical G1-S-G2-M cycles and availability of genetic tools (Garcia et al., 2007; Lee and Orr-Weaver, 2003). The first thirteen cell cycles of *Drosophila* embryogenesis involve nearly synchronous nuclear divisions driven by stockpiles of maternally expressed mRNA and protein (Foe et al., 1993). These rapid cycles (~ten minutes in length) consist of oscillating S-M (DNA replication-mitosis) phases without intervening gap phases or cytokinesis. Minimal gene transcription occurs during this developmental stage, so cell cycles are regulated by post-transcriptional mechanisms. At cycle 14, the embryo cellularizes and initiates zygotic transcription at the midblastula transition (MBT).

We report here the identification and characterization of a *Drosophila* maternal-effect lethal mutant that we have named "*no poles*" ("*nopo*"). Embryos from *nopo* females undergo mitotic arrest with acentrosomal, barrel-shaped spindles during syncytial divisions. Our results indicate this arrest is secondary to activation of a Chk2-mediated DNA checkpoint in early embryos. We show that NOPO, a predicted E3 ubiquitin ligase, interacts with an E2 component, BEN. *ben* females are sterile, producing embryos with *nopo*-like defects. We propose that BEN-UEV1A and NOPO function together as an E2-E3 complex required for genomic integrity during *Drosophila* embryogenesis.

RESULTS

The *nopo* phenotype in the early embryo

We previously screened the maternal-effect lethal subset of the Zuker collection to identify genes that regulate S-M cycles of *Drosophila* early embryogenesis (Koundakjian et al., 2004; Lee et al., 2003; Rickmyre et al., 2007). We identified an allele (*Z1447*) of a gene that we have named "*no poles*" (*nopo*) based on the phenotype of acentrosomal mitotic spindles in mutant-derived embryos (see below). $nopo^{Z1447}$ females are completely sterile (Table 2.1). DNA staining of egg chambers of $nopo^{Z1447}$ females revealed no obvious oogenesis defects, and the presence of polar bodies in their unfertilized eggs indicated that meiosis was completed (data not shown).

We found that $nopo^{Z1447}$-derived embryos undergo mitotic arrest during syncytial divisions with none developing to cellularization or gastrulation (Tables 2.1,2.2; data not shown). Nuclei are unevenly spaced (compare Fig. 2.1B to A), and centrosome

Table 2.1. *nopo* allelic series and transgenic rescue

Temperature (˚C)	Genotype	Cortical (%)[a]	Gastrulation (%)[b]	Hatch rate (%)
25	Wild type	92	91	89
	nopo^{SZ3004}	62	65	12
	nopo^{SZ3004}*/Df(2R)Exel7153*	67	12	0
	nopo^{Z1447}	72	0	0
	nopo^{Z1447}*/Df(2R)Exel7153*	68	0	0
	pCaSpeR4-CG5140/+; *nopo*^{Z1447}	95	92	87
	nopo^{Exc142}	15	0	0
	nopo^{Exc142}*/Df(2R)Exel7153*	7	0	0
	pCaSpeR4-CG5140/+; *nopo*^{Exc142}	93	90	83
18	Wild type	97	97	89
	nopo^{SZ3004}	78	86	76
	nopo^{Z1447}	67	1	0

Embryos collected from females of the indicated genotypes were fixed for DNA and tubulin staining (see methods for details).

[a]Percent of embryos that develop to cycle 10 or beyond. For each genotype, at least 200 embryos (2-2.5 hour) were scored.

[b]Percent of embryos that develop to initiation of gastrulation or beyond. For each genotype, at least 200 embryos (3-4 hour) were scored.

duplication before telophase is occasionally evident (Fig. 2.1F), both consistent with failed mitotic divisions. *nopo* spindles are barrel-shaped, lack tubulin foci, and have misaligned chromosomes (Fig. 2.1D-F; Table 2.2); lack of tubulin foci correlates with loss of centrosomes at the poles as revealed by staining for Centrosomin, a core component (Fig. 2.1I,J; Li and Kaufman, 1996). ~10% of *nopo* spindles are tripolar (Fig. 2.1K; Table 2.2). Bipolar *nopo* spindles often appear wider and to contain more than the wild-type complement of chromosomes (compare Fig. 2.1E and C). Similar results were obtained for $nopo^{Z1447}$ in trans to *Df(2R)Exel7153*, which deletes *nopo* (Table 2.1).

nopo encodes a RING domain-containing protein

We identified *CG5140* as the *nopo* gene using a combination of genetic mapping and molecular biology approaches (Fig. 2.2A; see methods for details). A wild-type copy of *CG5140* carried as a transgene fully restored fertility to *nopo* females (Table 2.1), confirming that *CG5140* is, indeed, the *nopo* gene. *nopo* encodes a predicted protein of 435 amino acids containing an N-terminal RING domain (Fig. 2.2B; Saurin et al., 1996). The putative mammalian homolog of NOPO was named "TRAF-interacting protein (TRIP)" based on its ability to bind tumor necrosis factor (TNF) receptor-associated factors (TRAFs) (Lee et al., 1997). Mammalian TRIP was recently demonstrated to have RING-dependent E3 ubiquitin ligase activity in an auto-ubiquitination assay (Besse et al., 2007). *Drosophila* NOPO and human TRIP are 20% identical and 34% similar overall with 47% identity and 65% similarity in their RING domains. Importantly, $nopo^{Z1447}$ causes a glutamic acid to lysine change in the RING domain at position 11 of the

Table 2.2. The *nopo* phenotype is suppressed by *mnk*

Genotype	M.I.[a]	Mitotic spindle defects (%Spindles)[b]				Gastrulation (%Embryos)[e]	Hatch rate (%)
		Bipolar spindles			Tripolar spindles		
		Abnormal centrosome#		Barrel-Shaped			
		Decreased[c]	Increased[d]				
Wild type	0.63	<1	0	<1	<1	100	89
nopo^{Z1447}	0.95	62	13	64	10	0	0
mnk^{6006}	0.64	1	<1	1	<1	99	80
mnk^{6006} *nopo*^{Z1447}	0.68	<1	<1	<1	<1	77	0

Embryos collected from females of the indicated genotypes were used to determine hatch rates or were fixed for DNA and tubulin staining (see methods for details).

[a]Mitotic Index (M.I.) = %Embryos in mitosis/Total number of embryos. >300 embryos were scored per genotype. Chromosome condensation and the presence of a mitotic spindle were used as the criteria for mitosis.

[b]All mitotic spindles (>500 total) in a single focal plane were scored using at least 25 embryos per genotype.

[c]Spindles with centrosomal detachment at one or both poles.

[d]Spindles with >1 centrosome per pole (one or both poles). Telophase spindles were not scored because centrosome duplication occurs during this phase in the early embryo.

[e]Stained embryos (3-4 hours) were scored for development to initiation of gastrulation (or beyond). >200 embryos were scored per genotype.

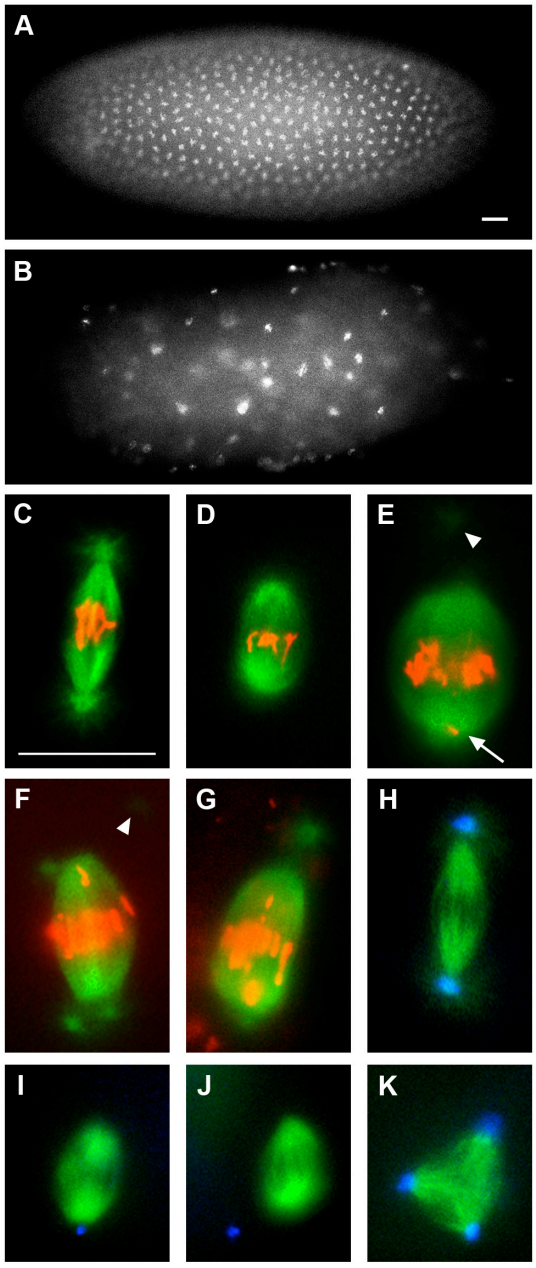


Figure 2.1. The *nopo* phenotype. Representative syncytial embryos and mitotic spindles in embryos from wild-type or *nopo*^{Z1447} females. (A,B) Staining of *nopo*-derived embryos reveals developmental arrest with condensed, unevenly spaced DNA (B) compared to wild type (A). (C-G) Microtubules are in green and DNA in red. (C) Wild-type spindle. (D-F) Shortened, barrel-shaped *nopo* spindles with detached centrosomes and misaligned chromosomes. Arrowheads, detached centrosomes out of focal plane; arrow, DNA at pole. Metaphase-like spindle with two centrosomes per pole (F) reveals asynchrony of nuclear and centrosome cycles. (G) Similar defects in *nopo*^{Exc142}/*Df(2R)Exel7153*-derived embryo. (H-K) Microtubules are in green and centrosomes in blue. (H) Wild-type spindle. (I,J) *nopo* spindles with detached and/or missing centrosomes. (K) Tripolar *nopo* spindle. Bars, 20 μm.

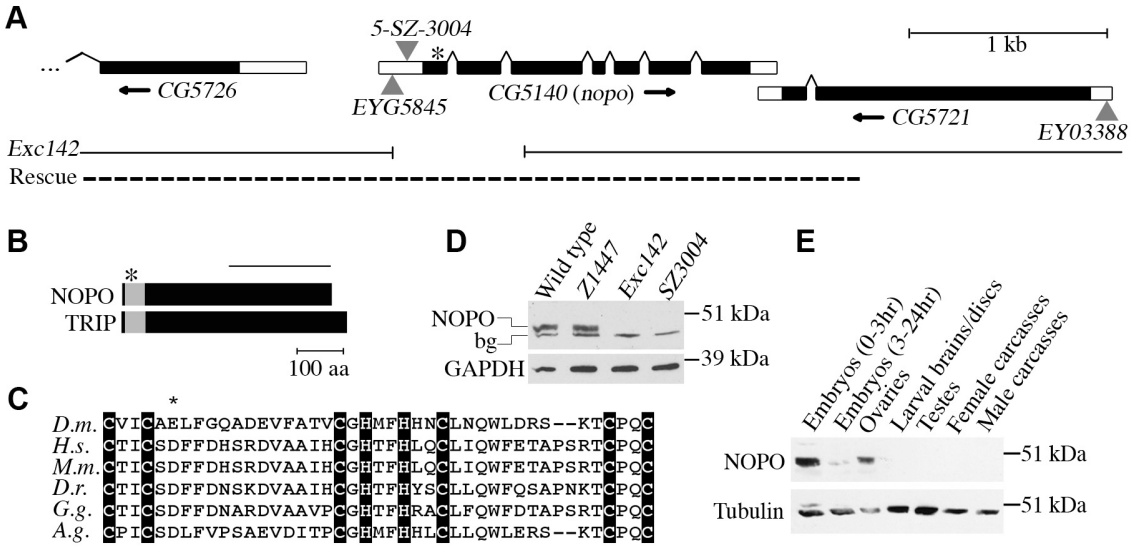


Figure 2.2. *CG5140* is the *nopo* gene. (A) *nopo* structure. Coding regions are represented by black boxes, 5'- and 3'-UTRs by white boxes, splicing events by lines. Arrows indicate transcription direction. Asterisk marks position of E11K mutation in $nopo^{Z1447}$. Triangles represent *P*-elements. *EYG5845* imprecise excision generated $nopo^{Exc142}$ (gap represents deleted region). Dashed line represents genomic region used to create rescue construct. (B) Comparison of *Drosophila* NOPO and human TRIP. Gray boxes represent RING domains. Asterisk marks mutation in $nopo^{Z1447}$. Line indicates NOPO region used for antibody production. (C) Alignment of RING domains of putative NOPO/TRIP homologs in *Drosophila melanogaster*, *Homo sapiens*, *Mus musculus*, *Danio rerio*, *Gallus gallus*, and *Anopheles gambiae*. Residues 6-46 of *Drosophila* NOPO are shown. Critical RING-domain cysteines and histidines are highlighted. Asterisk marks residue mutated in $nopo^{Z1447}$. (D,E) NOPO immunoblots. (D) NOPO levels in embryos (1-2 hours) of wild-type or *nopo* females. Anti-NOPO antibodies recognize specific band of NOPO's predicted size (48 kDa) and non-specific band (bg). (E) NOPO developmental Western. Loading control: anti-GAPDH or anti-α-tubulin.

predicted protein, a residue that is invariantly negatively charged across species (Fig. 2.2C).

nopo is maternally provided and essential solely in the early embryo

To gain further insights into *nopo*'s functions, we obtained additional alleles. *5-SZ-3004* (abbreviated as *SZ3004*) and *EYG5845* are *P*-element insertions in the 5'-UTR of *nopo* (Fig. 2.2A). $nopo^{SZ3004}$ females have decreased embryonic hatch rates that are completely restored by precise *P*-element excision (Table 2.1; data not shown). $nopo^{SZ3004}$ is weaker than $nopo^{Z1447}$ based on the percentage of mutant-derived embryos that develop to gastrulation and embryonic hatching, and its phenotype is strongly temperature-dependent. We generated a null allele of *nopo* (*Exc142*) via imprecise excision of *EYG5845* (Fig. 2.2A; see methods). $nopo^{Exc142}$ adults are viable and appear normal except that females are sterile, producing embryos with the *nopo* phenotype (Fig. 2.1G); a *CG5140* transgene fully restored fertility (Table 2.1). $nopo^{Exc142}$ is stronger than $nopo^{Z1447}$ with 15% versus 72%, respectively, of their embryos reaching cortical divisions, suggesting that $nopo^{Z1447}$ has residual function.

We assessed NOPO levels in mutant embryonic extracts by immunoblotting using anti-NOPO antibodies that we generated (Fig. 2.2D). Consistent with NOPO's predicted size (435 residues), these antibodies recognize a ~48 kDa band in wild-type embryos that is absent in $nopo^{Exc142}$-derived (null) embryos. NOPO was not detected in $nopo^{SZ3004}$-derived embryos, although we occasionally observe trace amounts (data not shown). Wild-type levels of NOPO were found in $nopo^{Z1447}$-derived embryos, suggesting that the E11K mutation alters NOPO's function, but not its stability.

We assessed NOPO levels throughout *Drosophila* development (Fig. 2.2E). NOPO is abundant in ovaries and early (0-3 hour) embryos; trace amounts are present in older (3-24 hour) embryos. We did not detect NOPO in larval brains, imaginal discs, testes, or adult carcasses lacking germline tissues. Subsequent experiments, however, revealed roles for NOPO outside of early embryonic development, suggesting that our antibodies might not be sufficiently sensitive to detect its expression during other stages (see below). Using the UAS/Gal4 system, we found NOPO overexpression in the female germline causes severely reduced egg-laying and hatch rates, whereas broad overexpression of NOPO in somatic cells causes lethality, suggesting that NOPO levels must be tightly regulated (data not shown).

The *nopo* phenotype is suppressed by mutation of the checkpoint kinase MNK (Chk2)

In *Drosophila* syncytial embryos, mitotic entry with incompletely replicated or damaged DNA triggers a Chk2-mediated protective mechanism known as centrosomal inactivation (Sibon et al., 2000; Takada et al., 2003). This damage-control system senses DNA defects and elicits localized changes in spindle structure that block mitotic progression, presumably to prevent propagation of defective DNA. We previously reported that embryos from *microcephalin* (*mcph1*) females arrest in mitosis with acentrosomal, barrel-shaped spindles similar to those that we now observe in *nopo*-derived embryos (Rickmyre et al., 2007). We demonstrated that these *mcph1* defects were suppressed by mutation of *maternal nuclear kinase* (*mnk*), also known as *loki*, which encodes *Drosophila* Chk2 (Abdu et al., 2002; Brodsky et al., 2004; Masrouha et

al., 2003; Xu et al., 2001). *mnk* nulls exhibit increased sensitivity to ionizing radiation but are viable and fertile. Suppression of *mcph1* by *mnk* revealed that centrosomal inactivation significantly contributes to the *mcph1* phenotype.

To determine if the mitotic defects of *nopo*-derived embryos are, like those of *mcph1*, due to Chk2-mediated centrosomal inactivation, we created lines doubly mutant for *nopo* and *mnk*. The *nopo* phenotype of acentrosomal, barrel-shaped mitotic spindles was strongly suppressed by *mnk* as evidenced by restoration of normal spindles with attached centrosomes (Fig. 2.3A-C; Table 2.2). DNA defects, however, are common in embryos from *mnk nopo* females, particularly during cortical divisions. We frequently observe abnormal DNA aggregates, some of very large size, shared by more than one spindle (Fig. 2.3D,E).

Like *mcph1*, we found that the developmental arrest of *nopo* mutants is suppressed by *mnk* (Fig. 2.3F,G; Table 2.2). In contrast to *nopo*-derived embryos, which arrest in syncytial divisions, most embryos from *mnk nopo* females complete syncytial divisions, cellularize, and arrest with aberrant morphology upon initiation of gastrulation. Cellularized embryos from *mnk nopo* females contain unusually large DNA masses within irregularly sized cells compared to wild type. Thus, *mnk* suppresses the spindle/centrosomal defects and developmental arrest of *nopo* mutants, but DNA defects appear to accumulate.

Based on these findings, we propose that *nopo* is required for preservation of genomic integrity during syncytial embryogenesis. Lack of *nopo* activity leads to occurrence of DNA defects, which then trigger Chk2-mediated centrosomal inactivation, thereby causing widespread mitotic arrest and blockade of embryonic development.

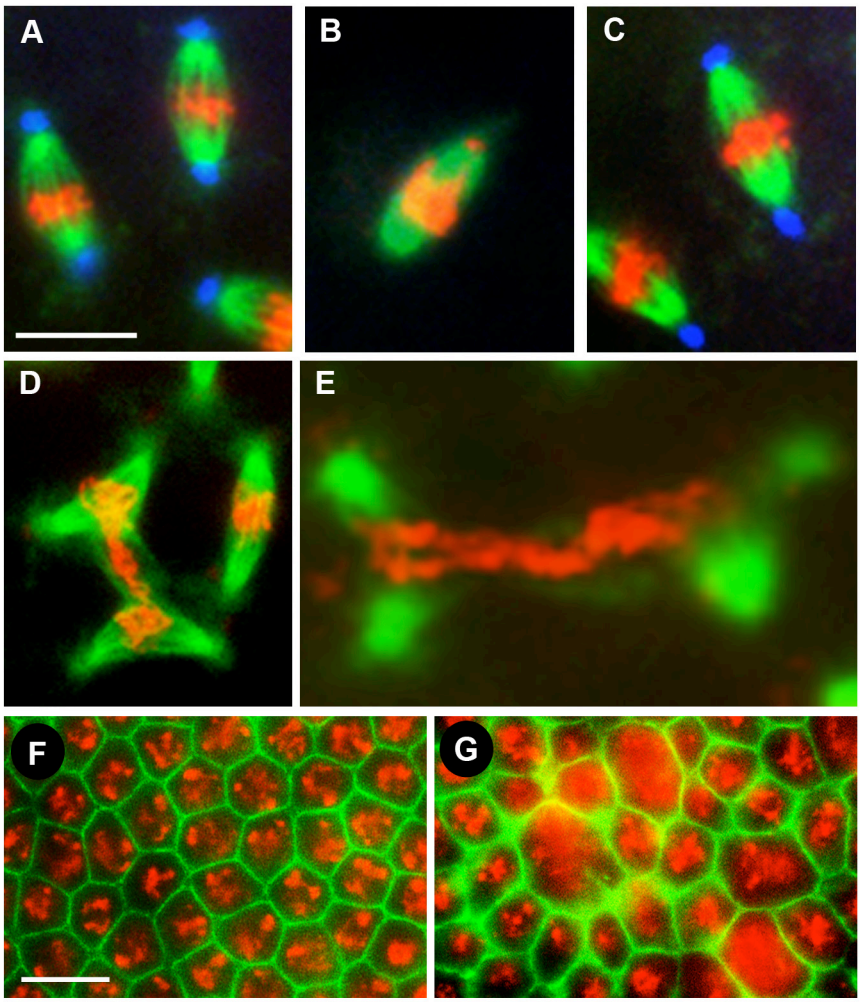


Figure 2.3. Suppression of *nopo* by *mnk*. (A-E) Representative mitotic spindles in syncytial embryos from wild-type (A), *nopo*^{Z1447} (B) and *mnk nopo*^{Z1447} females (C-E). (A-C) Microtubules are in green, DNA in red, and centrosomes in blue. *nopo* (B) is suppressed by *mnk* as evidenced by restoration of elongated spindles with attached centrosomes (C). (D,E) Microtubules are in green and DNA in red. Aberrant mitotic figures with DNA shared by two spindles in *mnk nopo*-derived embryos. (F,G) Cellularized embryos (2-3 hours). Actin is in green and DNA in red. Developmental arrest of *nopo* is suppressed by *mnk*. Cellularized *mnk nopo*^{Z1447}-derived embryos with large DNA masses (G) compared to wild type (F). Bars, 10 μm (A-E) or 20 μm (F,G).

Mutation of *mnk* (*Chk2*) allows further nuclear divisions and developmental progression in *nopo*-derived embryos despite accumulation of extensive DNA defects that eventually lead to their arrest at the onset of gastrulation.

nopo-derived embryos exhibit decreased interphase length

The DNA-replication checkpoint mediated by MEI-41 and Grapes, orthologs of ATR and Chk1, respectively, is developmentally activated in late syncytial embryos of *Drosophila* (Sibon et al., 1999; Sibon et al., 1997). Checkpoint activation, which may be triggered by titration of a maternal replication factor, leads to inhibitory phosphorylation of Cdk1 and gradual slowing of mitotic entry, presumably to allow sufficient time to complete DNA replication. At MBT (cycle 14), the first G2 gap phase is introduced. Embryos from *mei-41* or *grapes* (*grp*) females fail to lengthen interphases of late syncytial cycles and are thought to enter mitosis without completing DNA replication; a secondary damage-control system, Chk2-mediated centrosomal inactivation, then becomes operational (Sibon et al., 2000; Takada et al., 2003).

Mitotic entry with incompletely replicated DNA can cause Chk2-mediated centrosomal inactivation in syncytial embryos (Sibon et al., 2000; Takada et al., 2003). Control mechanisms to ensure completion of DNA replication prior to mitosis may be particularly critical during rapid S-M cycles. Oscillating Cdk1-Cyclin B activity plays a key role in coordinating these cycles (Edgar et al., 1994; Su et al., 1998). S-M transitions appear to be controlled by Cyclin B levels prior to cycle 10 and by both Cyclin B levels and a DNA-replication checkpoint in cycles 10-13 (Ji et al., 2004; Sibon et al., 1997).

Using a previously described approach, we monitored timing of nuclear envelope breakdown and reformation in cycles 11-13 by differential interference contrast (DIC) microscopy to test whether *nopo* mutants, like *mei-41* and *grp*, fail to lengthen interphase (Fig. 2.4; Rickmyre et al., 2007; Takada et al., 2007). Live imaging of *nopo*-derived embryos during cortical divisions was not feasible because the majority arrest either before or during these cycles (Table 2.1; data not shown); yolk proteins obscure the interior nuclei of precortical embryos, making imaging of these earlier divisions technically difficult. We analyzed timing of cortical cell cycles in embryos from *mnk nopo* females (lacking a Chk2-mediated checkpoint) because they develop further than *nopo*-derived embryos; we reasoned that primary defects in cell-cycle kinetics due to *nopo* mutation would still be apparent. In support of this line of reasoning, embryos from *mnk grp* females have been shown to retain the cell-cycle timing defects of *grp*-derived embryos (Takada et al., 2007). Embryos from *mnk* $nopo^{Z1447}$ females exhibited significantly shorter interphases during cycle 11 (mean of 3.3 minutes) compared to wild type or *mnk* (4.8 or 5.4 minutes, respectively; Fig. 2.5A,B). Essentially identical results were obtained for *mnk* $nopo^{Z1447}$*/Df(2R)Exel7153* and *mnk* $nopo^{Z1447}$/$nopo^{Exc142}$ females. Importantly, interphase 11 length was restored by transgenic rescue. A shortened interphase 11 (2.7 minutes) was observed in *grp*-derived embryos, as expected. Unlike *grp*, however, *mnk nopo*-derived embryos exhibited normal cycle 12 and 13 interphase lengths.

Based on our observations of shorter cycle 11 interphases in *mnk nopo*-derived embryos, we infer that interphases of earlier (precortical) syncytial cycles may be relatively short in *nopo*-derived embryos. We hypothesize that DNA replication is not

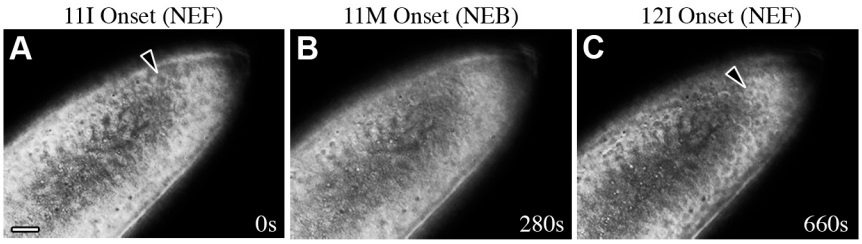
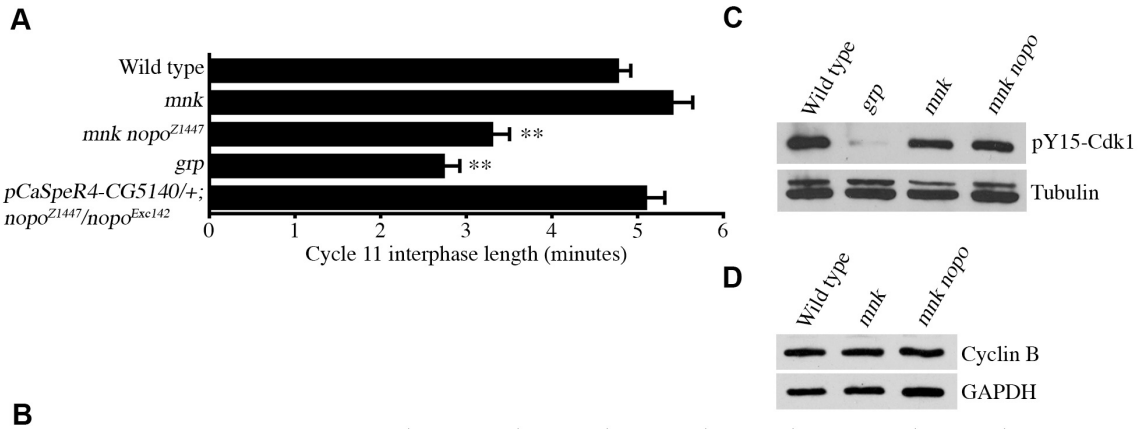


Figure 2.4. Cell-cycle timing of syncytial blastoderm divisions. Representative still DIC micrographs from an imaging sequence of a wild-type embryo illustrate the criteria used to score the onset of interphase and mitosis. Nuclear envelope "rings" (arrowheads) are readily visible at the cortex of the embryo during interphase but not mitosis. (A) Nuclear envelope formation (NEF) marks the onset of interphase 11. (B) Nuclear envelope breakdown (NEB) marks the onset of mitosis 11. (C) NEF marks the onset of the following interphase 12. Images were captured at 20-second intervals and cell-cycle timing determined by counting frame numbers between NEF and NEB. Timing data were independently validated by a second observer. Strict temperature control (22.0±0.5°C) was maintained throughout the experiment as monitored by a sensor on the microscope stage; wild-type and mutant embryos were alternately analyzed in every recording session as further control. Time (s, seconds) elapsed from the onset of cycle 11 is indicated for each micrograph. Bars, 10 μm.



B

Genotype	11I	11M	12I	12M	13I	13M	n
Wild type	4.8±0.2	6.1±0.4	6.9±0.3	7.7±0.4	13.3±0.6	7.5±0.4	10
mnk	5.4±0.2	6.2±0.5	6.7±0.3	7.5±0.8	13.4±0.4	8.0±0.3	5
mnk nopo^{Z1447}	3.3±0.2**	5.9±0.4	5.9±0.4	6.5±0.3	12.3±0.5	7.0±0.5	9
mnk nopo^{Z1447}/mnk Df(2R)Exel7153	3.3±0.2**	7.3±0.5	6.5±0.3	8.1±0.5	12.3±1.0	9.2±0.7	9
mnk nopo^{Z1447}/mnk nopo^{Exc142}	3.4±0.3**	7.2±0.6	6.0±0.4	7.5±0.1	13.3±0.7	8.5±0.5	5
pCaSpeR4-CG5140/+; nopo^{Z1447}/nopo^{Exc142}	5.1±0.2	6.3±0.3	6.6±0.4	7.6±0.5	11.2±0.8	8.1±0.4	7
grp	2.7±0.2**	6.8±0.4	4.8±0.6*	7.7±0.6	5.1±0.3**	9.1±0.4	5

Figure 2.5. Shortened cycle 11 interphase of *mnk nopo*-derived embryos. (A,B) Cell-cycle timing during cortical embryonic divisions. Bar graph (A) shows mean cycle 11 interphase lengths for various genotypes. Table (B) summarizes mean cycle 11-13 interphase (I) and mitosis (M) lengths. n, number of embryos. Error bars (A) and ± values (B) represent s.e.m. Single and double asterisks mark interphases significantly shorter than wild type (P-value<0.01 and <0.001, respectively). (C,D) Immunoblotting reveals normal pY15-Cdk1 (C) and Cyclin B (D) levels in *mnk nopo^{Z1447}*-derived embryos (1-2 hours). Control *grp*-derived embryos have reduced pY15-Cdk1. Loading control: anti-α-tubulin or anti-GAPDH.

completed during these truncated interphases, resulting in mitotic entry with unreplicated DNA, triggering of Chk2-mediated centrosomal inactivation, and mitotic arrest with failure of further embryonic development.

Most embryos from *nopo*-null females arrest prior to the onset at cycle 11 of a detectable DNA-replication checkpoint effect (Table 2.1; Crest et al., 2007). Thus, we reasoned that *nopo* is unlikely to regulate interphase length via *mei-41*/*grp*. Nonetheless, we tested intactness of the MEI-41/GRP-mediated DNA-replication checkpoint by assessing levels of Cdk1 inhibitory phosphorylation in *mnk nopo*-derived embryos and found them to be comparable to wild type (Fig. 2.5C). We also observed an intact DNA damage response by *nopo* larvae treated with hydroxyurea, a DNA replication inhibitor, or irradiation (Table 2.3). We observed no genetic interactions between *nopo* and *mei-41* (data not shown). We detected wild-type levels of Cyclin B and Cyclin A in *mnk nopo*-derived embryos and observed no genetic interactions between *nopo* and *cyclin B* (Fig. 2.5D; data not shown). These results suggest that *nopo* regulates the S-M transition independent of the MEI-41/GRP-dependent checkpoint and mitotic cyclin levels.

Drosophila NOPO and human TRIP co-localize to nuclear puncta in cultured mammalian cells

To determine the subcellular localization of NOPO, we used transfected mammalian cells because our anti-NOPO antibodies did not work for immunofluorescence, and epitope-tagged forms of NOPO expressed via transgenesis were not stable in *Drosophila* embryos (data not shown). We transfected HeLa cells with fluorescently tagged versions of *Drosophila* NOPO and human TRIP (candidate homolog

Table 2.3. Survival of *nopo* larvae after hydroxyurea treatment or irradiation

	Percent homozygotes[b]		Percent eclosion[c]	
Genotype	**-HU**	**+HU**	**0 Gray**	**10 Gray**
mei-41^{RT1}	62 (557)	3 (235)	82 (170)	3 (177)
nopo^{Z1447}	34 (562)	35 (587)	86 (107)	87 (112)
nopo^{Exc142}	n.d.[a]	n.d.	92 (149)	88 (129)
nopo^{Z1447}*/Df(2R)Exel7153*	n.d.	n.d.	98 (124)	89 (128)
nopo^{Exc142}*/Df(2R)Exel7153*	35 (440)	31 (397)	92 (210)	97 (167)
nopo^{Z1447}*/ nopo*^{Exc142}	n.d.	n.d.	99 (147)	99 (192)

[a]n.d., not determined.

[b]Sensitivity to hydroxyurea (HU). First instar larvae were grown on food minus or plus HU and allowed to develop. For each genotype, the ratio of homozygous mutant to total progeny is expressed as a percentage with total number of adult flies scored shown in parentheses. Expected percentages (based on Mendelian ratios) were 50% and 33% for *mei-41* and *nopo*, respectively.

[c]Sensitivity to irradiation. Third instar larvae were untreated or exposed to low-dose irradiation and allowed to develop. For each genotype, the ratio of eclosed adults to total pupae is expressed as a percentage with total pupae shown in parentheses.

of NOPO) and assessed their localizations by immunofluorescence microscopy. Whereas eGFP (control) is homogeneously distributed, eGFP-NOPO localizes to nuclear puncta in a majority of interphase cells (compare Fig. 2.6A to B); a similar pattern was observed for Myc-tagged NOPO (data not shown). mCherry-TRIP also exhibited a punctate distribution in nuclei (Fig. 2.6D). Co-expression of eGFP-NOPO and mCherry-TRIP in HeLa cells confirmed their essentially identical localization patterns, underscoring the likelihood that NOPO and TRIP are functional homologs (Fig. 2.6C-E).

CREST staining of HeLa cells expressing eGFP-NOPO revealed that NOPO/TRIP localizes to nuclear regions distinct from centromeres. To assess whether eGFP-NOPO localizes to nuclear puncta in a cell cycle-dependent manner, we immunostained transfected HeLa cells for PCNA or Cyclin A. We found that >99% of cells positive for eGFP-NOPO puncta were negative for insoluble PCNA foci in the nucleus, a marker of S-phase (Somanathan et al., 2001). In contrast, ~97% of cells positive for eGFP-NOPO puncta were positive for nuclear Cyclin A, a marker of both S and G2 phases (Girard et al., 1991). Taken together, these data indicate that eGFP-NOPO specifically localizes to nuclear puncta in transfected HeLa cells during G2 phase.

NOPO associates with BEN, an E2 heterodimer component

The presence of a RING domain in NOPO suggested that it might function as an E3 ubiquitin ligase (Lorick et al., 1999). In a high-throughput yeast two-hybrid screen (Giot et al., 2003), NOPO interacted with an E2 ubiquitin-conjugating enzyme, Bendless (BEN), the *Drosophila* homolog of Ubc13 (Muralidhar and Thomas, 1993; Oh et al., 1994; Zhou et al., 2005).

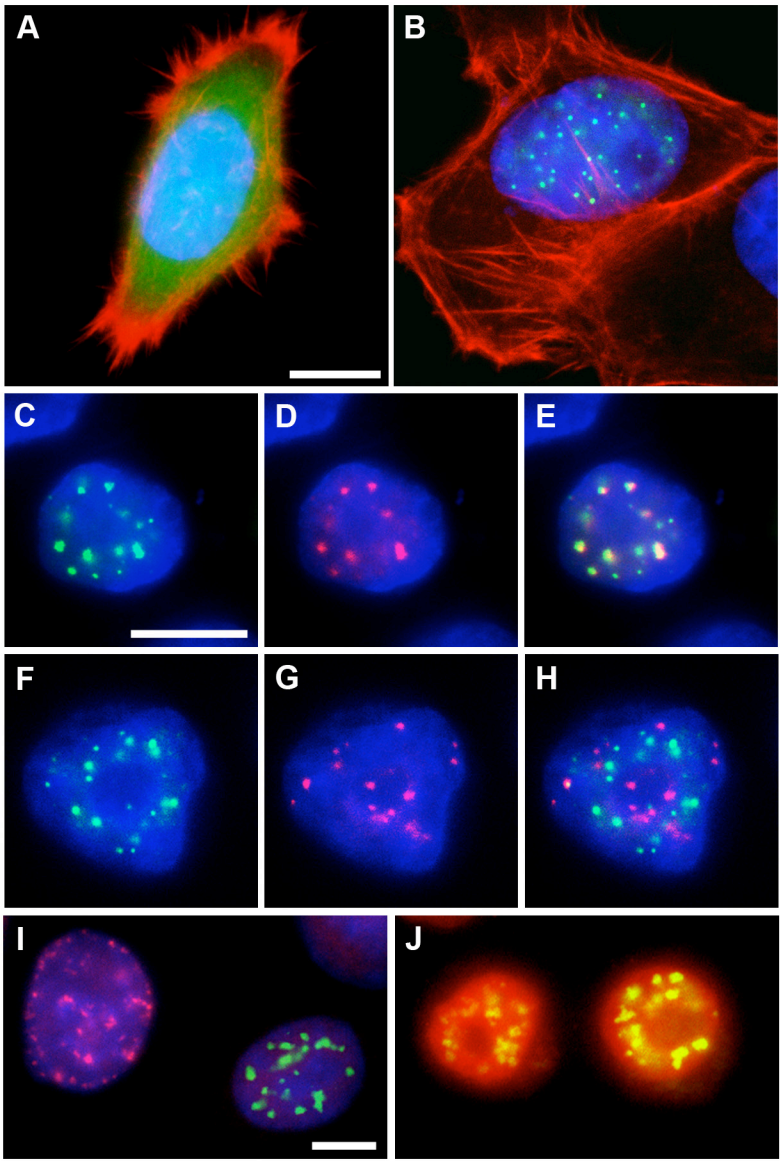


Figure 2.6. Nuclear localization of NOPO. Immunofluorescence microscopy of transfected HeLa cells. DNA is in blue. (A,B) eGFP is in green and actin in red. eGFP-*Drosophila* NOPO (B) localizes to nuclear puncta; eGFP (A) is homogeneously distributed. (C-E) eGFP is in green and mCherry in red. eGFP-*Drosophila* NOPO (C) and mCherry-human TRIP (D) co-localize in nuclear puncta (E, merge). (F-H) eGFP is in green and CREST in red. eGFP-NOPO (F) is not at centromeres (G) (H, merge). (I) Cells with GFP-NOPO puncta (green) are negative for PCNA puncta (red). (J) Cells with GFP-NOPO puncta (green) are positive for nuclear Cyclin A (red). Bars, 10 μm.

To confirm and extend these observations, we tested for interactions between combinations of wild-type and mutant NOPO and BEN proteins in a yeast two-hybrid assay (Fig. 2.7A). We used mutant NOPO and BEN forms encoded by *nopo*^{Z1447} (E11K in the RING domain; Fig. 2.2A-C) and *ben*^{1} (proline to serine change at position 97; Muralidhar and Thomas, 1993). We found that both wild-type and mutant NOPO self-interacts in this assay. When used as bait, wild-type BEN strongly interacts with wild-type NOPO; we occasionally observe weak interaction in the reverse direction (data not shown). In contrast, wild-type BEN and mutant NOPO do not interact, and mutant BEN interacts only marginally with wild-type or mutant NOPO.

We detected comparable levels of mutant and wild-type fusion proteins (both NOPO and BEN) in transformed yeast. Furthermore, *nopo*^{Z1447}-derived embryos have wild-type NOPO levels, and *ben*^{1} ovaries have wild-type BEN levels (Fig. 2.2D; data not shown). Thus, lack of two-hybrid interactions observed for mutant NOPO and BEN likely reflects changes in protein-protein interactions rather than decreased stability.

To obtain further evidence that NOPO and BEN interact, we compared the localization patterns of fluorescently tagged versions of these proteins in transfected HeLa cells. Both eGFP-NOPO and mCherry-TRIP accumulate in nuclear puncta (Figs. 2.6E, 2.7C). When transfected alone, eGFP-BEN distributes throughout cells with a perinuclear concentration but no obvious nuclear puncta (Fig. 2.7B). When eGFP-BEN and mCherry-TRIP were co-transfected, however, eGFP-BEN localized to nuclear puncta in the majority (56%) of cells positive for mCherry-TRIP nuclear puncta; furthermore, all puncta positive for eGFP-BEN were positive for mCherry-TRIP (Fig. 2.7D-F). These

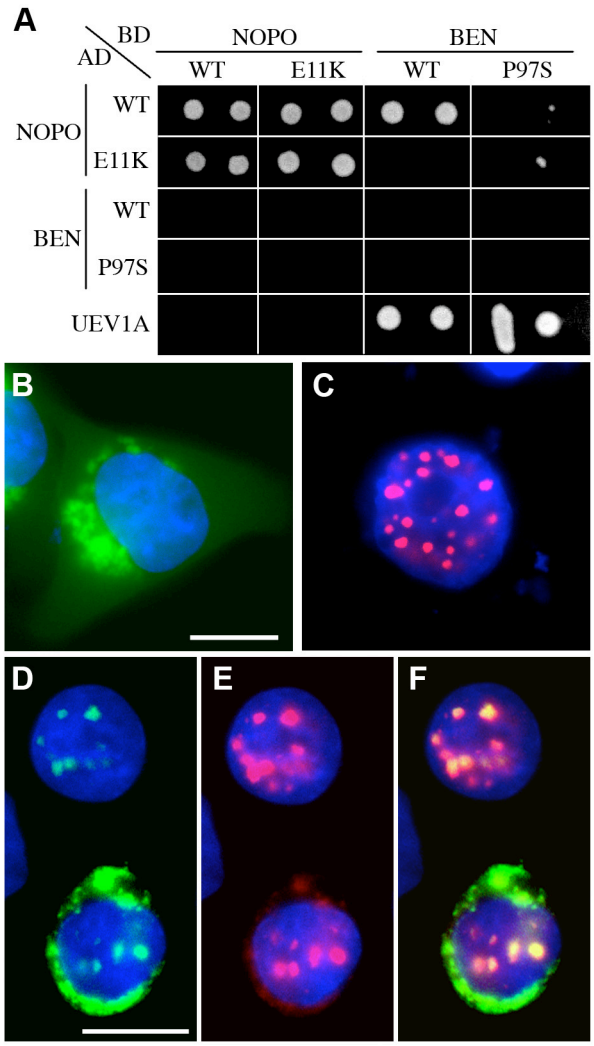


Figure 2.7. NOPO/TRIP, BEN, and UEV1A interactions and co-localization. (A) Yeast two-hybrid assay. Yeast cells expressing combinations of NOPO, BEN, and UEV1A fused to Gal4 DNA binding domain (BD, "bait") or activation domain (AD, "prey") were spotted onto selective media. Growth on SC-Trp-Leu-His media (shown) indicates physical interaction between fusion proteins. Wild-type and mutant versions of NOPO and BEN (E11K and P97S, respectively) were tested. A representative plate spotted in duplicate is shown; identical results were obtained for three independent $Trp^{+}Leu^{+}$ colonies per plasmid combination tested. (B-F) Immunofluorescence microscopy of transfected HeLa cells. eGFP-BEN is in green, mCherry-TRIP in red, and DNA in blue. eGFP-BEN (C) and mCherry-TRIP (D) localize distinctly when transfected alone. (D-F) Co-transfection of eGFP-BEN (D) with mCherry-TRIP (E) promotes its localization into nuclear puncta (F, merge). Bars, 20 μm.

data suggest that NOPO/TRIP can recruit BEN/Ubc13 to chromatin and provide further evidence for in vivo interactions between these proteins.

The E2 activity of Ubc13 has been shown in other systems to require heterodimerization with a UEV (ubiquitin-conjugating E2 enzyme variant) family member (Pickart, 2001). UEV proteins (Mms2p in budding yeast; Uev1A and Mms2 in mammals) resemble E2s but lack an active site cysteine (Broomfield et al., 1998; Sancho et al., 1998). Our BLAST searches revealed a single UEV homolog in *Drosophila* encoded by *Uev1A*. In our two-hybrid assay, UEV1A strongly interacted with wild-type and mutant BEN, but not with NOPO (Fig. 2.7A). Our attempts to detect BEN-NOPO complexes in *Drosophila* embryos were unsuccessful, however, possibly due to transience of this interaction (data not shown). Our yeast two-hybrid data suggest that NOPO's RING domain interacts directly with BEN to promote formation of a UEV1A-BEN-NOPO (E2-E3) complex.

ben-derived embryos have *nopo*-like defects

ben was identified in a screen for *Drosophila* mutants with neuronal connectivity defects (Thomas and Wyman, 1982). Its yeast two-hybrid interaction with NOPO suggested that BEN might regulate embryonic development. We found that embryos from ben^{1} homozygotes or hemizygotes fail to develop, revealing a new function for *ben* (Table 2.4).

Immunostaining of ben^{1}-derived embryos revealed that they arrest early in syncytial development with a small number (1-8) of mitotic nuclei (Fig. 2.8; Table 2.4). Most (72%) ben^{1}-derived embryos contain one acentrosomal spindle. In *Drosophila*

Table 2.4. Quantification of defects in *ben*-derived embryos

Genotype	M.I.[a]	Hatch rate (%)	#Nuclei/Embryo (%Embryos) 1	2-8	>8	*nopo*-like spindles (%Embryos)[c]
Wild type	0.63	89	-	-	-	<1
ben^{1}	0.92	2[b]	72	12	16	80
ben^{1}*/Df(1)KA10*	0.80	0	40	43	17	50

Embryos collected from females of the indicated genotypes were used to determine hatch rates or were fixed for DNA and tubulin staining (see methods for details). At least 50 stained embryos were analyzed per genotype. Eggs/embryos in which DNA and tubulin were not visualized by staining were excluded from analysis.

[a]Mitotic Index (M.I.) = %Embryos in mitosis/Total number of embryos. Chromosome condensation and the presence of a mitotic spindle were used as the criteria for mitosis.

[b]For *ben*^{1} homozygotes, only 100 embryos were scored for hatching due to low egg-laying rates.

[c]Embryos in which the majority of mitotic spindles were barrel-shaped and/or lacking centrosomes were scored as being "*nopo*-like."

females, meiotic spindles lack centrosomes, which are provided by sperm (Foe et al., 1993). Several lines of evidence suggest that *ben*^{1} acentrosomal spindles are mitotic rather than meiotic. Their presence requires fertilization, and they are positioned deep within the egg interior where the first mitotic spindle resides (meiotic spindles are positioned near the cortex); furthermore, the presence of polar bodies indicates completion of meiotic divisions, and centrosomes are occasionally seen near the spindle (Fig. 2.8A-E; data not shown).

The spindle defects of *ben*^{1}-derived embryos strikingly resemble those of *nopo* mutants (compare Fig. 2.8H,I to G). *ben*^{1} spindles are often acentrosomal, barrel-shaped, variable in width, and have misaligned chromosomes; similar phenotypes were observed in *ben*^{1} hemizygotes (Fig. 2.8J,K; Table 2.4). We were unable to test whether Chk2-mediated centrosomal inactivation causes mitotic arrest in *ben*-derived embryos because doubly homozygous adults were not viable. Taken together, the yeast two-hybrid interactions, co-localization, and similar mutant phenotypes that we have observed suggest that BEN-UEV1A and NOPO function together as an E2-E3 complex required to preserve genomic integrity during early embryonic development in *Drosophila*.

Assessment of *ben*-mediated functions in *nopo* mutants

Because a given E2 can act in concert with multiple E3 ubiquitin ligases (Pickart, 2001), we sought to determine which of BEN's activities are mediated by NOPO. We assayed our *nopo* mutants for four additional biological functions previously ascribed to BEN. The *Drosophila* giant fiber system (GFS) is a simple neural circuit that mediates an escape response to visual stimuli (Allen et al., 2006). Because *ben* is required for proper

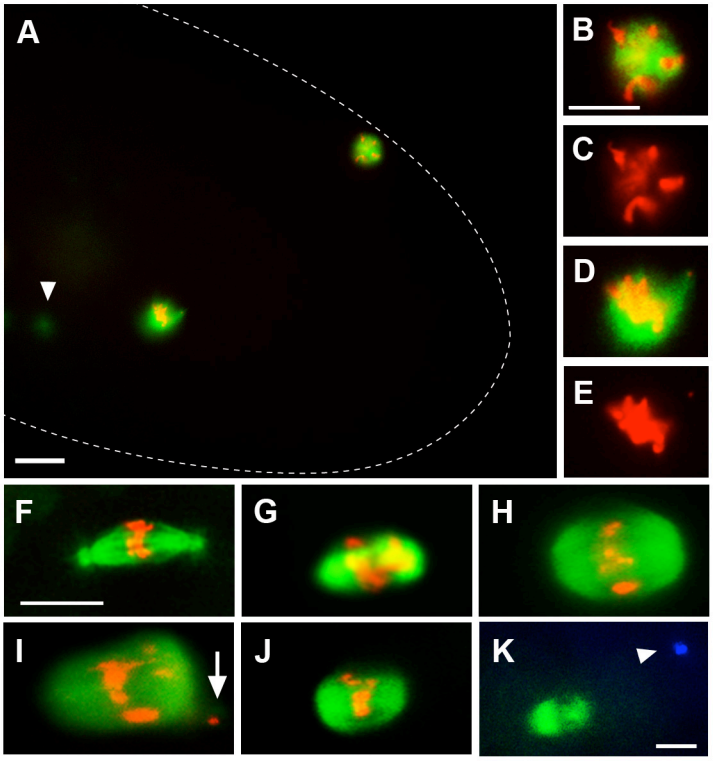


Figure 2.8. *ben* phenocopies *nopo*. Representative mitotic spindles in syncytial embryos from wild-type, *nopo*, and *ben* females. (A-J) Microtubules are in green and DNA in red. (A-E) Single mitotic spindle and polar body in ben^{1}-derived embryo. (A) Dashed line marks embryo outline. Arrowhead, detached centrosome out of focal plane. (B-E) Magnified images of polar body (B,C) and mitotic spindle (D,E) from A. (F-K) Mitotic spindles in embryos from wild-type (F), $nopo^{Z1447}$ (G), ben^{1} (H,I), and $ben^{1}/Df(1)HA92$ (J,K) females. *ben*-derived embryos exhibit *nopo* phenotypes, including barrel-shaped, acentrosomal spindles and displaced DNA (I, arrow). (K) Microtubules are in green and centrosomes in blue. $ben^{1}/Df(1)HA92$ spindle with detached centrosome (arrowhead). Bars, 20 μm (A) or 10 μm (B-K).

synaptic connectivity in the GFS, *ben* adults fail to elicit a normal jump response to a light-off stimulus (Thomas and Wyman, 1982; Thomas and Wyman, 1984). *ben* adults have also been reported to exhibit abnormalities in thoracic musculature and impaired mobility, and a role in innate immunity has been ascribed to *ben* (Edgecomb et al., 1993; Zhou et al., 2005).

We assessed intactness of the GFS of *nopo* flies by testing their jump response to visual stimuli and found them to be defective like *ben* flies (Fig. 2.9A; Oh et al., 1994; Thomas and Wyman, 1982). Unlike *ben*, however, *nopo* flies exhibited normal mobility in a climbing assay and had normal sites of attachment of tergal depressor of the trochanter (TDT) thoracic muscles to the scutellum (Fig. 2.9B,C). We tested the innate immune responses of *nopo* and *ben* males and found that both exhibited slightly reduced levels of diptericin induction after infection (Fig. 2.9D). These results suggest that BEN and NOPO act as an E2-E3 complex that regulates GFS synapse formation and innate immunity, whereas BEN regulates mobility and muscle attachment sites via a different E3 ligase; thus, NOPO mediates a subset of BEN's functions.

DISCUSSION

We propose a model in which NOPO, a RING domain-containing protein, interacts with the BEN-UEV1A heterodimer to form a functional E2-E3 ubiquitin ligase complex required during syncytial embryogenesis for genomic integrity, cell-cycle progression, and continuation of development (Fig. 2.10). In the absence of NOPO, lack of ubiquitination of yet unidentified NOPO targets results in truncation of S-phase and/or spontaneous DNA damage. Mitotic entry with unreplicated and/or damaged DNA

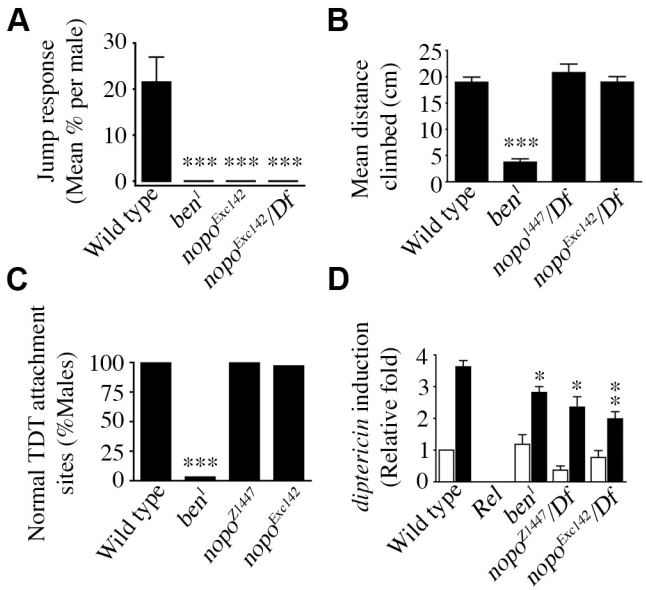


Figure 2.9. NOPO mediates a subset of BEN's functions. Wild-type, *ben*, and *nopo* males were compared using several assays. (A) Visually-mediated jump response assay. (B) Climbing assay. (C) Assessment of TDT muscle attachment sites. (D) Innate immunity assay. *diptericin* induction was measured after injection of buffer (white bars) or *E. coli* (black bars). *Relish* (*Rel*) flies with defective immune response were used as control. *Df* is *Df(2R)Exel7153*. Error bars represent s.e.m. Single, double, and triple asterisks represent *P*-values <0.05, <0.01, and <0.001, respectively.

triggers activation of a Chk2-mediated checkpoint that leads to changes in spindle morphology, mitotic arrest, and failure of *nopo*-derived embryos to develop to cellularization.

We favor a model in which NOPO regulates timing of S-M transitions in syncytial embryos to ensure that S-phase is of sufficient length to allow completion of DNA replication prior to mitotic entry. Inhibition of DNA replication in syncytial embryos (e.g. via aphidicolin injection) leads to chromatin bridging in subsequent mitoses and Chk2 activation, both of which occur in *nopo*-derived embryos, presumably due to mitotic entry with unreplicated chromosomes (Raff and Glover, 1988; Takada et al., 2003). The mechanism by which NOPO coordinates S-M transitions is unknown. Our data suggest that *nopo* may alter timing of these transitions independent of Cdk1-CyclinB, although localized changes in levels and/or activities of these regulators not detectable by immunoblotting of whole-embryo lysates could play a critical role. It is unclear why the MEI-41/GRP-dependent checkpoint, which appears to be functional in *nopo*-derived embryos, would not be sufficient to slow mitotic entry.

The punctate nuclear localization observed for NOPO and its human homolog, TRIP, expressed in HeLa cells may indicate a direct role for these proteins in regulation of chromatin structure. Furthermore, the G2 phase-specific localization that we observe for NOPO/TRIP in transfected HeLa cells may be consistent with a role for NOPO in slowing S-M transitions in syncytial embryos; in the absence of *nopo*, embryos that enter mitosis prematurely would likely do so without finishing DNA replication due to a lack of gap phases.

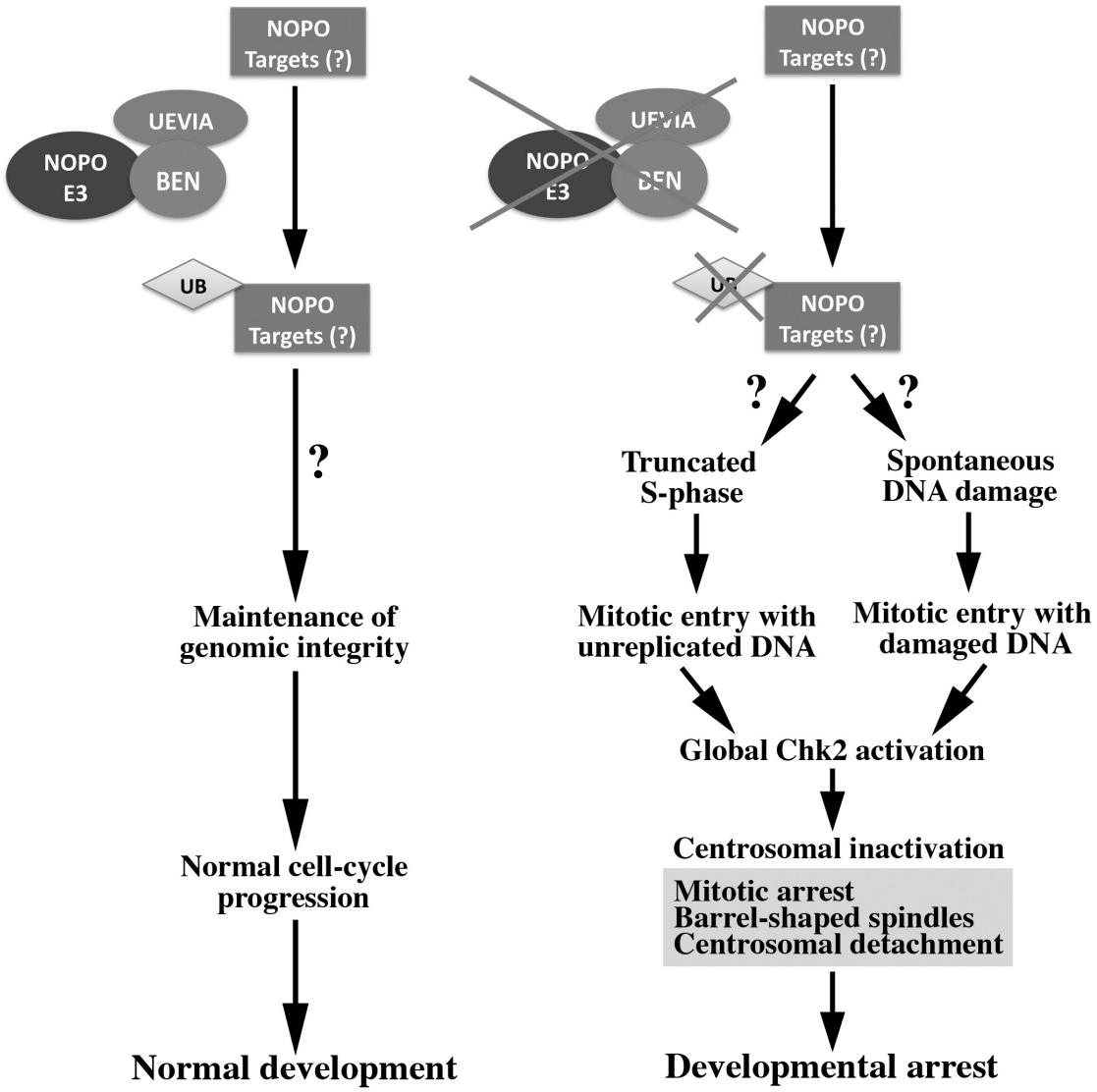


Figure 2.10. Model for NOPO's function in the early embryo. See text for details.

An alternative explanation for Chk2 activation in *nopo*-derived embryos is that they might incur elevated levels of spontaneous DNA damage. Syncytial embryos are considered to be unusual in that they activate Chk2 but not Chk1 in response to DNA damaging agents (Fogarty, et al., 1997; Sibon et al., 2000; Takada et al., 2007). Thus, spontaneous DNA damage would not be predicted to elicit the MEI-41/GRP-mediated replication checkpoint but would cause Chk2-dependent centrosomal inactivation during mitosis. Such a model would be consistent with the apparent lack of activation of the MEI-41/GRP-dependent checkpoint in *nopo*-derived embryos although it would not explain why interphase 11 is shortened.

We previously reported that syncytial embryos from *microcephalin* (*mcph1*) females undergo mitotic arrest with a phenotype similar to that described herein for *nopo* (Rickmyre et al., 2007). Like *nopo*, Chk2-mediated centrosomal inactivation causes mitotic arrest in embryos lacking *mcph1*. *nopo* and *mcph1* are unique among maternal-effect lethal mutants in which Chk2-mediated centrosomal inactivation has been reported (e.g. *grp*, *mei-41*, *wee1*) in that their phenotypes appear to be more severe: centrosomes typically detach from spindles, and mitotic arrest occurs earlier, during precortical syncytial divisions (Rickmyre et al., 2007; Sibon et al., 2000; Stumpff et al., 2004; Takada et al., 2003). The underlying defects in *nopo* and *mcph1* mutants may be distinct, however, because *mnk mcph1*-derived embryos exhibit normal cycle 11 interphase length, which are truncated in *mnk nopo*-derived embryos (Rickmyre et al., 2007). Furthermore, we detect no genetic interaction between *nopo* and *mcph1* (J.L. Rickmyre, J.A. Merkle, and L.A. Lee, unpublished).

Mammalian TRIP was identified in a yeast two-hybrid screen for tumor necrosis factor (TNF) receptor-associated factor (TRAF) interactors (Lee et al., 1997). TRAFs transduce signals from members of the tumor necrosis factor (TNF)/tumor necrosis factor receptor (TNFR) superfamily, which elicit diverse cellular responses in the immune and inflammatory systems (Hehlgans and Pfeffer, 2005). TRIP has been reported to inhibit TRAF2-mediated NF-kB activation; the RING domain of TRIP, however, was not required for inhibition (Lee et al., 1997). In contrast, our analysis of $nopo^{Z1447}$ indicates that this motif is essential for NOPO function in *Drosophila* embryogenesis, likely by mediating its interactions with E2 components as has been shown for other E3 ligases (Passmore and Barford, 2004). *Drosophila* Eiger (TNF ligand) and Wengen (TNF receptor) play roles in dorsal closure, neuroblast divisions, and the response to fungal pathogens (Kauppila et al., 2003; Schneider et al., 2007; Wang et al., 2006). A role for TNF signaling in early *Drosophila* embryogenesis has not been reported to our knowledge.

TRIP was recently reported to be an essential factor in mice (Park et al., 2007). TRIP-deficient mice die soon after implantation due to defects in early embryonic development. Compared to wild-type littermates, $TRIP^{-/-}$ embryos are smaller in size with reduced cell number. TRAF2 does not appear to be required until later in development, suggesting that TRIP has TRAF2-independent roles in early embryos (Nguyen et al., 1999). It will be interesting to see whether mammalian TRIP, by analogy to *Drosophila* NOPO, is required for genomic integrity during embryonic development.

Our data support a model in which NOPO ubiquitin ligase acts in concert with BEN-UEV1A heterodimers to regulate *Drosophila* syncytial embryogenesis. The yeast

two-hybrid interaction and co-localization of NOPO and BEN led us to identify an unanticipated role for BEN in early embryogenesis and additional roles for NOPO in synapse formation and innate immunity. While the spindle defects of *ben*-derived embryos are strikingly similar to those of *nopo*, they typically arrest earlier in syncytial development, suggesting that another E3 ligase that requires BEN may function in parallel with NOPO. Although *nopo* egg chambers appear normal, we have not ruled out a possible requirement for BEN-UEV1A-NOPO complexes during oogenesis; some defects in *nopo*- and *ben*-derived embryos could be a secondary consequence of previous defects during oogenesis.

K63-linked ubiquitin chains are thought to act as non-proteolytic signals (e.g. affecting protein localization and/or interactions), whereas K48-linked ubiquitin chains have established roles in targeting proteins for proteasome-mediated degradation (Pickart and Fushman, 2004). BEN-UEV1A E2 homologs in budding yeast (Ubc13-Mms2p) mediate K63-linked polyubiquitination of PCNA during postreplicative repair (Andersen et al., 2005). In mammalian cells, the E2 heterodimer Ubc13-Mms2 mediates DNA damage repair, while Ubc13-Uev1A promotes NF-κB activation; both E2 complexes regulate these processes by mediating K63 ubiquitin chain assembly on target proteins. We propose that BEN-UEV1A-NOPO (E2-E3) complexes mediate assembly of K63-linked ubiquitin chains on proteins that preserve genomic integrity in early *Drosophila* embryogenesis.

CHAPTER III

YEAST TWO-HYBRID SCREEN IDENTIFIES TRIP INTERACTORS

INTRODUCTION

In order to monitor the integrity of the genome and to prevent the transmission of genetic errors to daughter cells during cell division, the cell employs a complex network of regulatory proteins that sense DNA damage and either repair it or block cell-cycle progression. DNA damage can occur at any stage of the cell cycle, exists in many forms and may result from many different sources. Disruption of the DNA damage response (DDR) may result in unrepaired DNA damage and uncontrolled proliferation (reviewed by Zhou and Elledge, 2000), which may result in pathologies such as cancer can result.

In animal cells, the DDR, which may result in a permanent cell-cycle arrest or cell death, is largely controlled by the tumor suppressor p53. p53, a transcription factor that is activated by ATM and ATR, is required to turn on transcription of several target genes involved in the prevention of cell-cycle progression and the initiation of apoptosis (Hirao et al., 2000). p53 activity is positively regulated by the acetyltransferase p300 and the kinases ATR, ATM and Chk2. p53 is also negatively regulated, mostly by the ubiquitin-proteasome system and the E3 ubiquitin ligase MDM2 (Oliner et al., 1993). A major target of p53 transcriptional activity is the Cdk inhibitor p21, which inhibits Cdk/cyclin and prevents cell-cycle progression at G1/S (Shivji et al., 1994). If the DDR is prolonged, many cells initiate p53-mediated apoptosis. Because p53 is such a critical player in the DDR of animal cells, it is not surprising that it is the single most frequently mutated

protein in cancer cells (Soussi, 2003).

MDM2 (murine double minute 2), one of the E3 ubiquitin ligases for p53, is a RING domain-containing oncoprotein. Under normal conditions, MDM2 ubiquitylates and shuttles p53 out of the nucleus for degradation by the proteasome (Oliner et al., 1993; Lee and Gu, 2010; Marine and Lozano, 2010). In the presence of DNA damage or cellular stress, the tumor suppressor $p19^{ARF}$ blocks MDM2-mediated p53 ubiquitylation and nuclear export by interacting with and relocalizing MDM2 to the nucleolus (Wang et al., 2006). MDM2 can no longer ubiquitylate and promote the degradation of p53 once it is sequestered to the nucleolus. The nucleolus is the site of ribosomal RNA biosynthesis, but other roles for the nucleolus in cell-cycle progression and DNA repair suggest this nuclear compartment is multifaceted (Boisvert et al., 2007). MDM2 also has several p53-independent roles: MDM2 can affect cell-cycle checkpoints, DNA and centrosome replication, and DNA-repair pathways independent of p53 (Bouska and Eischen, 2009). MDM2 is essential for viability and development, as MDM2 homozygous mutant mice are embryonic lethal (Bond et al., 2005).

LZAP (also named CDK5RAP3 or C53) is a tumor suppressor that plays important roles in multiple cell processes, including the DDR and NF-κB signaling (Wang et al., 2006; Wang et al., 2007). LZAP is deposited maternally and highly conserved from plants to humans. Overexpression of LZAP promotes Cdk1 activity and the nuclear accumulation of cyclin B (Jiang et al., 2009). LZAP also interacts with and inhibits Chk1, thereby promoting Cdk1 activation and mitotic entry. These and other data suggest that LZAP plays a critical role in modulating the G2/M DNA damage checkpoint. Recently, the zebrafish homolog of LZAP was shown to be involved in cell cycle

progression and embryonic development (Liu et al., 2011). Zebrafish embryos injected with a morpholino designed to inhibit LZAP translation exhibit a delay in G2/M and arrest during early embryogenesis due to a failure to initiate epiboly. Preliminary studies on LZAP homozygous mutant mice are consistent with the findings in zebrafish; LZAP null mice die during early embryonic development (Liu et al., 2011). These data suggest critical roles for LZAP during cell cycle progression and development.

Above, I described a *Drosophila* maternal effect-lethal mutant *no poles* (*nopo*) (Merkle et al., 2009). Embryos from *nopo* females undergo mitotic arrest during the rapid S-M cycles of syncytial embryogenesis. Checkpoint kinase 2 (Chk2) plays a unique checkpoint function in the early *Drosophila* embryo as it is locally activated in response to DNA damage or incomplete replication. Our genetic data indicate that Chk2 is activated in *nopo* mutants, suggesting that NOPO plays a role in preserving genomic integrity during early *Drosophila* embryogenesis. We also showed that syncytial embryos lacking NOPO exhibit a significantly shorter interphase 11 as compared to wild type, suggesting that *nopo* mutants may enter mitosis prior to the completion of DNA replication, thereby triggering Chk2 activation (Merkle et al., 2009).

The candidate RING domain-containing E3 ubiquitin ligase, NOPO, is homologous to human TRAF-interacting protein (TRIP). Tumor necrosis factor receptor (TNFR)-associated factors (TRAFs) are key adaptor molecules in the TNF-signaling pathway that regulates cell proliferation, activation, differentiation and apoptosis (reviewed by Ha et al., 2009). However, TRIP's physiological role in TNF signaling is unclear (Lee et al., 1997; Regamey al., 2003). Although substrates of mammalian TRIP

have not been reported to date, the mouse TRIP homolog is a functional E3 ligase based on its *in vitro* auto-ubiquitylation activity (Besse et al., 2007).

Protein ubiquitylation plays an important role in many cellular processes, including protein processing, cell cycle control, chromatin remodeling, DNA repair, and membrane trafficking (Liu and Chen, 2011; Broemer and Meier, 2009; Le Bras et al., 2011; Acconcia et al., 2009; Hershko, 1997; Al-Hakim et al., 2010; O'Connell and Harper, 2007). Ubiquitylation involves the covalent linkage of one or more ubiquitin molecules to another protein. The resulting modification may alter its fate in a number of ways, including destruction by the 26S proteasome, a change in subcellular location, or a change in protein-protein interactions (Glickman and Ciechanover, 2002). E3s serve as the specificity factors in the multi-step process of ubiquitylation, as they bind both the E2 conjugating enzyme (E2) and the substrate to be ubiquitylated.

To identify potential ubiquitylation substrates of the E3 ligases NOPO and TRIP and elucidate the mechanism by which NOPO/TRIP promotes genomic stability, we performed a yeast two-hybrid screen for TRIP interactors. We report here the results of this screen and the subsequent follow-up on several interesting interactors. In particular, we focus in this chapter on the interaction of mammalian TRIP with MDM2 (an E3 ubiquitin ligase for p53) and the tumor suppressor LZAP. Identification and subsequent characterization of the interaction of NOPO/TRIP with members of the Y family of DNA polymerases will be the focus of the next chapter (Chapter 4).

RESULTS

TRIP interactors identified via yeast two-hybrid screen

RING-containing E3 ligases often interact directly with their substrates (Deshaies and Joazeiro, 2009). To identify potential substrate(s) of NOPO and hTRIP, we performed a yeast two-hybrid screen. Human TRIP cDNA was cloned into pGBKT7 vector (Matchmaker III from Clontech), transformed into yeast, and expression was verified by Western blot analysis (data not shown). A HeLa cell cDNA library was used to screen for clones that interact with yeast expressing the pGBKT7-hTRIP bait plasmid. Sequence analysis of 190 clones revealed the identity of 67 non-redundant hits (Figure 3.1; Table 3.1). We classified about 77% of these hits as common false positives (including mitochondrial, ribosomal and uncharacterized proteins) (Fig. 3.2). We further narrowed down the list of hits to pursue by prioritizing those that would help to place NOPO/TRIP within a molecular framework, in particular to elucidate the mechanism by which NOPO promotes genomic integrity during early embryonic development in *Drosophila*. This shortened list includes proteins involved in cell cycle, DNA damage and/or protein ubiquitylation (Table 3.2).

Confirmation of TRIP/NOPO interactors via yeast two-hybrid assays

To confirm interactions between TRIP and candidate hits from the primary screen, we individually retested their interactions with TRIP via yeast two-hybrid assays by cloning each full-length cDNA into pGADT7 and transforming the prey plasmids into yeast. We found that TSG101, MAGOH and MDM2 reproducibly interact with TRIP in

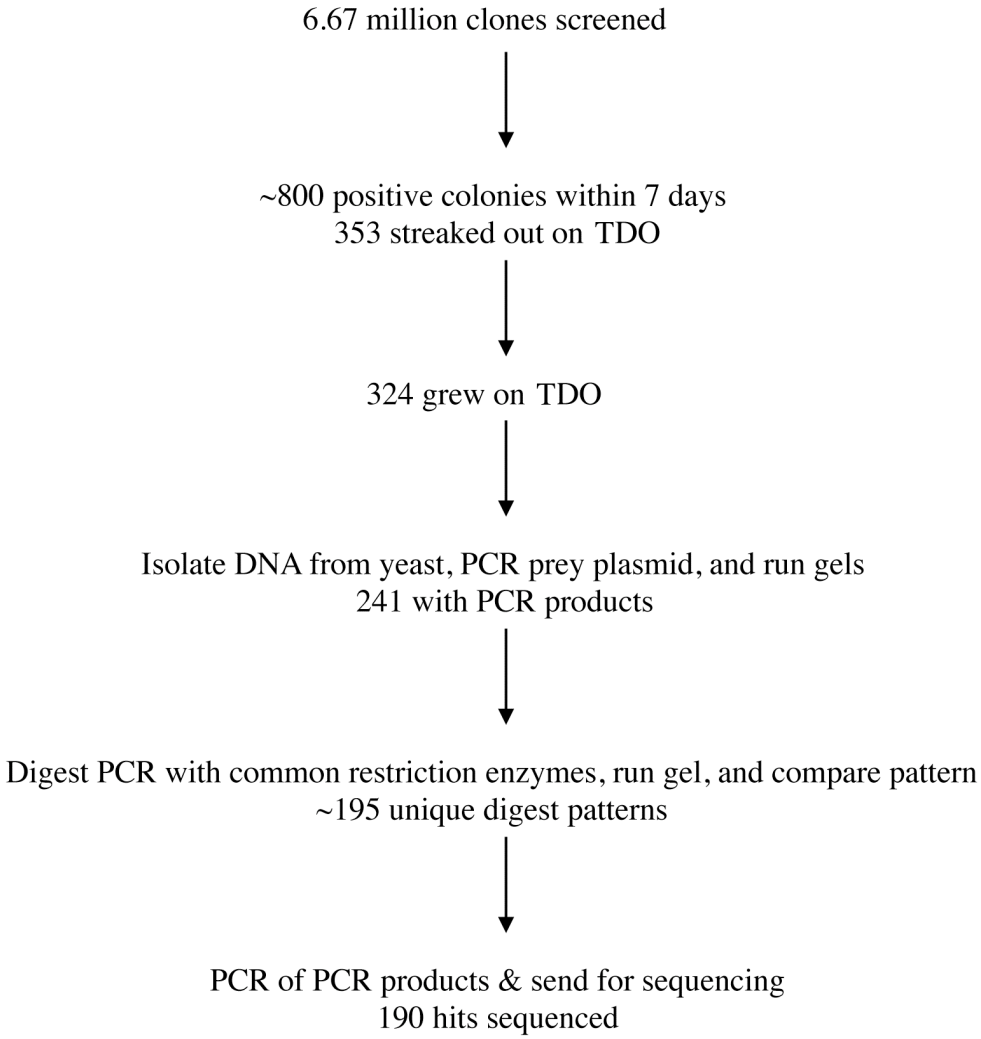


Figure 3.1: Yeast two-hybrid screen for TRIP interactors. Human *TRIP* cDNA was subcloned into pGBKT7 to encode a hybrid TRIP protein containing the DNA-binding domain of Gal4. Yeast cells of the AH109 strain were transformed with pGBKT7-hTRIP and mated with yeast cells pretransformed with a HeLa cDNA library (Clontech). Transformants were selected on minimal media lacking His, Leu and Trp (triple dropout, TDO) selection medium. Details of the follow-up for this screen are described in the text.

Table 3.1. List of hits from yeast two-hybrid screen using hTRIP as bait and HeLa cDNA library as prey.

Gene symbol	Gene ID	Gene name	# of times isolated in screen
AIP	9049	aryl hydrocarbon receptor interacting protein	1
AKR1B1	231	aldo-keto reductase family 1, member B1 (aldose reductase)	2
ARID2	196528	AT rich interactive domain 2 (ARID, RFX-like)	1
ATP5A1	498	ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit 1	1
CCT7	10574	chaperonin containing TCP1, subunit 7 (eta)	1
CD63	967	CD63 antigen	1
CDK5RAP3	80279	CDK5 regulatory subunit associated protein 3	1
CNPY3	10695	canopy 3 homolog (zebrafish)	1
COPS6	10980	COP9 constitutive photomorphogenic homolog subunit 6 (*Arabidopsis*)	1
DCTN1	1639	dynactin 1 (p150, glued homolog, *Drosophila*)	1
DENND4C	55667	DENN/MADD domain containing 4C	1
EBP	10682	emopamil binding protein (sterol isomerase)	1
EEF1A1	1915	eukaryotic translation elongation factor 1 alpha 1	2
EIF4A1	1973	eukaryotic translation initiation factor 4A, isoform 1	1
ENO1	2023	enolase 1, (alpha)	2
ETFA	2108	electron-transfer-flavoprotein, alpha polypeptide	8
FAM20C	56975	family with sequence similarity 20, member C	1
FTL	2512	ferritin, light polypeptide	8
GMPS	8833	guanine monphosphate synthetase	1
GNB2L1	10399	guanine nucleotide binding protein (G protein), beta polypeptide 2-like 1	1
IFRD2	7866	interferon-related developmental regulator 2	2
JAK3	3718	Janus kinase 3	1
MAGOH	4116	mago-nashi homolog, proliferation-associated	1

		(*Drosophila*)	
MDM2	4193	Mdm2 p53 binding protein homolog (mouse)	1
MESDC2	23184	mesoderm development candidate 2	3
NDUFB10	68342	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 10	1
NDUFV1	4723	NADH dehydrogenase (ubiquinone) flavoprotein 1	1
NINL	22981	ninein-like	1
PCOLCE	5118	procollagen C-endopeptidase enhancer	1
PEA15	8682	phosphoprotein enriched in astrocytes 15	1
PEX12	5193	peroxisomal biogenesis factor 12	1
POLK	51426	polymerase (DNA directed) kappa	1
POLR2E	5434	polymerase (RNA) II (DNA directed) polypeptide E	5
POLR2G	5436	polymerase (RNA) II (DNA directed) polypeptide G	1
RASSF7	8045	Ras association (RalGDS/AF-6) domain family (N-terminal) member 7	1
RNASET2	8635	ribonuclease T2	1
RPL11	6135	ribosomal protein L11	4
RPL31	6160	ribosomal protein L31	3
RPS14	6208	ribosomal protein S14	1
RPS7	6201	ribosomal protein S7	2
SAMD11	148398	sterile alpha motif domain containing 11	1
SART1	9092	squamous cell carcinoma antigen recognized by T cells	1
SAT1	6303	spermidine/spermine N1-acetyltransferase 1	3
SBF2	81846	SET binding factor 2	1
SC4MOL	6307	sterol-C4-methyl oxidase-like	1
SERINC3	10955	serine incorporator 3	1
SFRS7	6432	splicing factor, arginine/serine-rich 7	1
SOD2	6648	superoxide dismutase 2, mitochondrial	1
TAF1	6872	TAF1 RNA polymerase II, TATA box binding protein (TBP)-associated factor	2

TARBP1	6894	TAR (HIV-1) RNA binding protein 1	1
TBC1D5	9779	TBC1 domain family, member 5	1
TINAGL1	64129	tubulointerstitial nephritis antigen-like 1	1
TSG101	7251	tumor susceptibility gene 101	1
TXNDC9	10190	thioredoxin domain containing 9	1
UNC84A	23353	unc-84 homolog A (*C. elegans*)	2
USP15	9958	ubiquitin specific peptidase 15	1
VDAC2	7417	voltage-dependent anion channel 2	2
VIL1	7429	villin 1	1
VKORC1	79001	vitamin K epoxide reductase complex, subunit 1	1
WWC3	55841	WWC family member 3	2
YBX1	4904	Y box binding protein 1	2
ZBED4	9889	zinc finger, BED-type containing 4	1
ZDHHC6	64429	zinc finger, DHHC domain containing 6	2

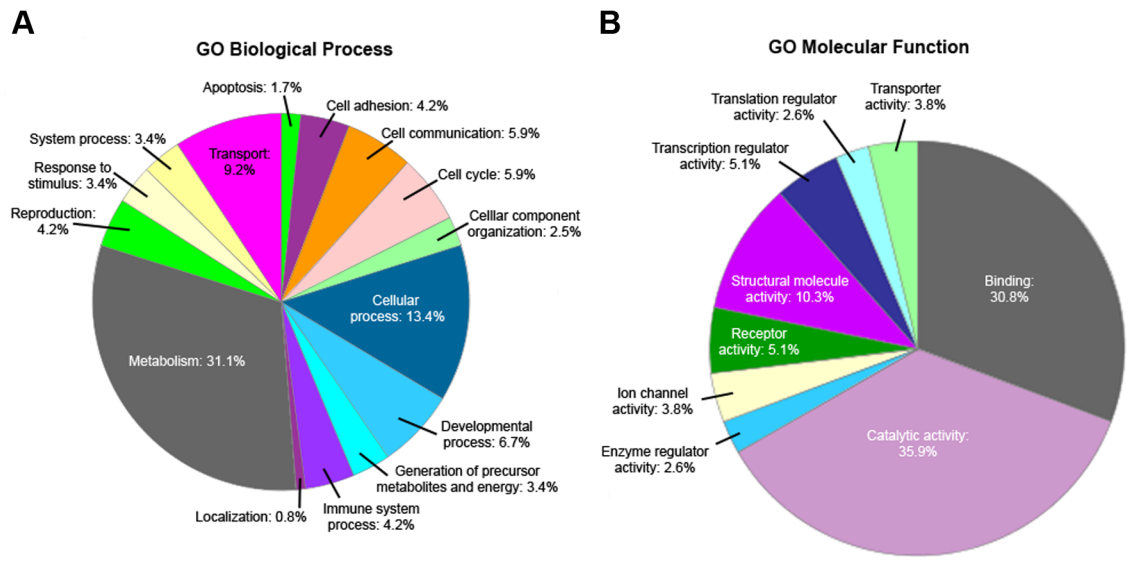


Figure 3.2: Gene Ontology (GO) classification of yeast two-hybrid hits. GO categories were assigned using PANTHER (www.pantherdb.org). (A) Pie chart of yeast two-hybrid hits classified into GO biological process categories. (B) Pie chart of yeast two-hybrid hits classified into GO molecular function categories. The percentage of genes classified in each category is indicated. Genes may be assigned to multiple categories by this program.

Table 3.2: Highest priority hits obtained from TRIP yeast two-hybrid screen.

Gene name:	Known functions:
TSG101 (tumor susceptibility gene 101)	E2 conjugating enzyme variant *Drosophila* homolog, *erupted*, involved in JAK-STAT signaling
MDM2 (murine double minute 2, homolog)	E3 ubiquitin ligase for p53 Sequestered to the nucleolus by pARF in the presence of DNA damage
POLK (DNA polymerase kappa)	Y-family DNA polymerase involved in translesion synthesis during S-phase
CDK5RAP3/LZAP (CDK5 regulatory subunit associated protein 3)	Role in NF-κB signaling & tumor progression Knockdown delays Cdk1 activation & mitotic entry
MAGOH (mago nashi homolog)	Regulates transcriptional activation of STAT3 Required for germline stem cell differentiation in *Drosophila*
USP15	Deubiquitylase (DUB) for IκBα

this assay (Fig. 3.3A). We also tested the interaction of NOPO and *Drosophila* homologs of some of the high-priority hits via yeast two-hybrid assays. We found that NOPO interacts with MAGO (MAGOH homolog) and CG20391 (LZAP homolog) (Fig. 3.3B). These data indicate that TSG101, MAGOH, MDM2 and LZAP are all TRIP/NOPO interactors by yeast two-hybrid assay and thereby represent potential substrates of these E3 ubiquitin ligases.

Confirmation of MDM2 and LZAP as TRIP interactors in cultured mammalian cells

To determine whether these interactions occur in cultured cells, we coexpressed MYC-tagged TRIP with FLAG-tagged human MDM2 or LZAP in HeLa cells. We found that FLAG-MDM2 and FLAG-LZAP were both coimmunoprecipitated with MYC-TRIP (Fig. 3.4A,B). These interactions were also confirmed in the reverse direction: MYC-TRIP was present in immunoprecipitates of the FLAG-tagged proteins (Fig 3.4C).

We previously reported the subcellular localization of eGFP-TRIP (N-terminally tagged) transiently expressed in HeLa cells to nuclear puncta during G2 phase (Merkle et al., 2009). Another group reported that endogenous TRIP localizes to the nucleolus (Zhou and Geahlen, 2009). We confirmed their results by transiently transfecting TRIP-mCherry (C-terminally tagged) into Hela cells and colocalizing TRIP with a nucleolar marker; however, we also observed TRIP-mCherry localized to nuclear puncta in these transfected cells (Fig 3.5; data not shown).

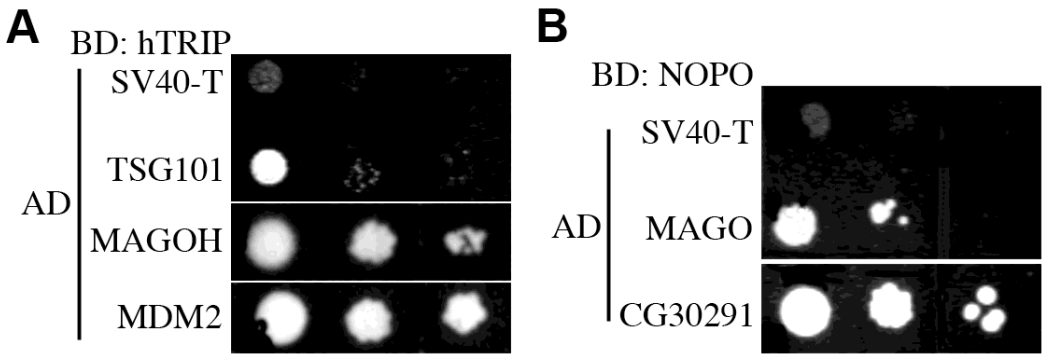


Figure 3.3: Confirmation of TRIP and NOPO interactors in yeast two-hybrid assays. Yeast cells expressing TRIP or NOPO baits fused to the DNA-binding domain of Gal4 (BD) and full-length preys fused to the Gal4 activation domain (AD) were spotted onto selective media. Physical interactions were tested by spotting diluted cells onto triple dropout (TDO) medium *(-Trp, -Leu, -His*) and scoring growth after 3 days at 30°C. SV40 T-antigen (SV40-T) was used as a negative control prey for all bait constructs. (A) Full-length human TRIP was used as bait. TRIP interacts with TSG101, MAGOH, and MDM2. (B) Full-length NOPO was used as bait. NOPO interacts with MAGO and CG30291.

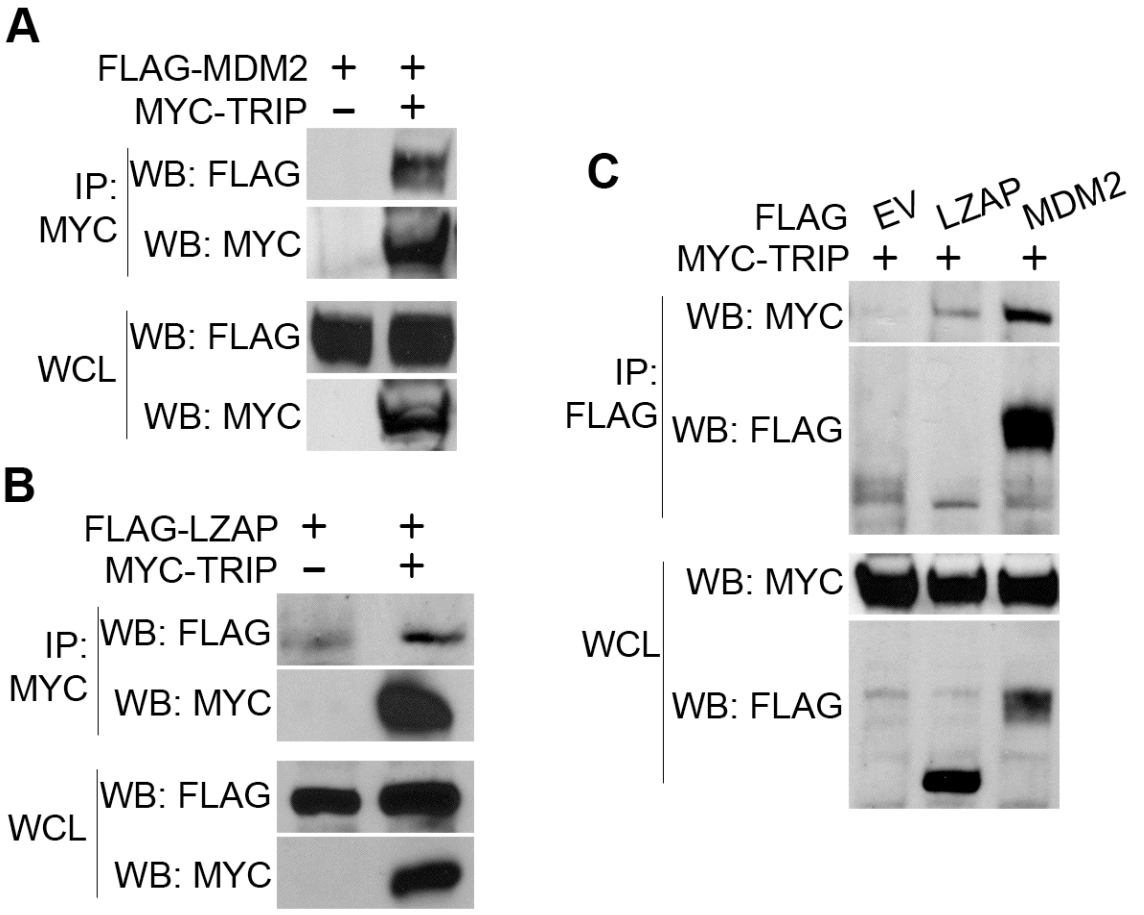


Figure 3.4: TRIP interacts with MDM2 and LZAP in cultured mammalian cells. (A-B) HeLa cells were co-transfected with MYC-tagged TRIP or empty vector and FLAG-tagged MDM2 (A) or LZAP (B). MYC-TRIP complexes were immunoprecipitated (IP) from cell lysates and resolved by SDS-PAGE/immunoblotting. FLAG-MDM2 (A) and FLAG-LZAP co-immunoprecipitated with MYC-TRIP. (C) HeLa cells with co-transfected with FLAG-tagged LZAP, MDM2, or empty vector (EV) and MYC-TRIP. FLAG-tagged protein complexes were immunoprecipitated from cell lysates and resolved by SDS-PAGE/immunoblotting. MYC-TRIP co-immunoprecipated with FLAG-MDM2 and FLAG-LZAP. Immunoblotting of whole cell lysates (WCL) used in all IP assays is shown.

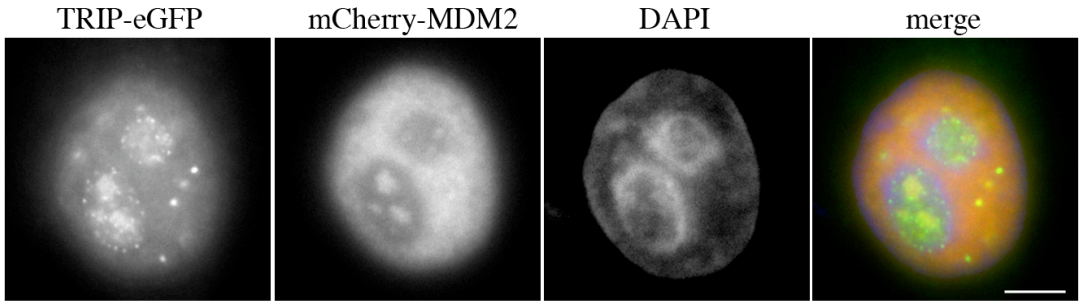


Figure 3.5: MDM2 co-localizes with TRIP in cultured mammalian cells. Immunofluorescence microscopy of HeLa cells co-transfected with TRIP-eGFP (green) and mCherry-MDM2 (red). DNA is in blue. TRIP-eGFP localizes to nuclear puncta and nucleoli mCherry-MDM2 localizes to the nucleus and the nucleolus. The merged image shows co-localization of TRIP and MDM2 to nucleoli. Bars, 10 μm.

TRIP co-localizes with MDM2 to the nucleus and nucleolus

Provided the reported endogenous localization of MDM2 to the nucleus and nucleolus, we questioned whether TRIP might interact with MDM2 at these subcellular sites (Chen and Chen, 2003). We compared the localization patterns of fluorescently tagged versions of these proteins in transfected HeLa cells and found that TRIP colocalized with MDM2 in transfected HeLa cells (Fig. 3.5). These data support a model in which TRIP interacts with MDM2 in the nucleolus.

DISCUSSION

Although the function of TRIP at the nucleolus is unknown, this nuclear compartment has been shown to be a site at which proteins are sequestered and held inactive (Boisvert et al., 2007). The nucleolus is the site of ribosome biosynthesis, although many other roles have been reported. Our identification of several cDNAs encoding ribosomal proteins in our yeast two-hybrid screen may actually be due to TRIP's subcellular localization rather than representing false-positive interactions. Interestingly, MDM2 is sequestered to the nucleolus in the presence of DNA damage so as to block ubiquitylation and degradation of the tumor suppressor p53. Based on TRIP's localization and interaction with the E3 ubiquitin ligase MDM2, we favor a model in which TRIP ubiquitylates and sequesters its substrates to the nucleolus under certain cellular conditions (i.e. cell-cycle stage or genotoxic stress). By analogy to the regulation of MDM2 by $p19^{ARF}$, when TRIP's substrate(s) is needed, we predict its release from the nucleolus is triggered.

We also identified in our screen proteins associated with cellular metabolism, apoptosis and NF-κB signaling. These hits could be associated with TRIP's role in TNF signaling. Although TRIP's physiological role in TNF signaling is unclear, the TNF-signaling pathway is linked to cell proliferation, activation, differentiation and apoptosis (Lee et al., 1997; Regamey et al., 2003). When TRIP is knocked down in keratinocytes, cells exhibit an NF-κB-independent G1/S phase cell-cycle arrest (Almeida et al., 2011). There is also evidence that mammalian TRIP is essential during development; homozygous mutant mouse embryos die shortly after implantation with proliferation defects and excessive cell death (Park et al., 2007). This early embryonic developmental arrest occurs prior to when TNF signaling is first required, suggesting that TRIP has critical roles outside of its proposed TNF-signaling functions (Gerhart, 1999). This role for TRIP may be linked to MDM2 and/or LZAP, as MDM2 and LZAP mutant mice similarly die in early embryonic development. It will be interesting to see if any of these proposed roles for TRIP in apoptosis and cell proliferation involve its interaction with LZAP and/or MDM2. Further investigation is likely to reveal new roles for TRIP in TNF-dependent and –independent processes.

It will also be important to determine if TRIP's interactions with LZAP and MDM2 are that of an E3 ligase and its substrates. Further experiments are necessary in order to better understand TRIP's role in regulating LZAP and MDM2. Because all of these proteins have been implicated in cell-cycle progression, genome maintenance, and development, uncovering the mechanisms by which these proteins functionally interact will be informative. Characterization of the *Drosophila* LZAP homolog, CG30291, has not been reported; similarly, identification of a functional MDM2 homolog in *Drosophila*

has yet to be reported (Sekelsky et al., 2000). We are particularly interested in future efforts to investigate the potential roles of NOPO in regulating MDM2 and LZAP homologs during early *Drosophila* embryogenesis.

CHAPTER IV

REGULATION OF TRANSLESION DNA POLYMERASES BY THE E3 UBIQUITIN LIGASES TRIP/NO POLES

INTRODUCTION

The early embryonic development of organisms such as *Drosophila melanogaster*, *Xenopus laevis*, *Danio rerio* and *Caenorhabditis elegans* are characterized by a rapid progression through the cell cycle (O'Farrell et al., 2004; Budirahardja and Gönczy, 2009). These rapid cell cycles of early embryogenesis may have evolved as a survival strategy due to the exposed environment in which the animals develop. The early embryonic cell cycles of *Drosophila* consist of oscillating rounds of DNA replication and mitosis with no intervening gap phases or cytokinesis. Cell-cycle regulators are provided maternally and slowly depleted until the mid-blastula transition (MBT).

Most eukaryotic cells employ highly regulated responses to damaged DNA. In the presence of DNA lesions, the PI3-like kinases ATR and ATM and their effector kinases, Chk1 and Chk2, are activated, thereby triggering a signaling cascade to respond to and repair the damage. The *Drosophila* syncytial embryo lacks a G2 phase, so it does not allow time for canonical cell cycle checkpoints to repair damaged DNA. During early embryogenesis in *Drosophila*, *mei-41*, the ATR homolog, and *grapes* (*grp*), the Chk1 homolog, are required to slow the late syncytial cell cycles (11-13) in order to introduce a G2 phase at cellularization (Sibon et al., 1999; Fogarty et al., 1994; Sibon et al., 1997). Embryos from *mei-41* or *grp* mutant females fail to lengthen interphase in the late

syncytial cycles and enter mitosis with incompletely replicated DNA, thus highlighting a unique role of DNA damage machinery during development.

Just as the eukaryotic cell possesses many mechanisms to repair damaged DNA, cells also possess mechanisms to temporarily tolerate DNA damage until the DNA repair machinery can effectively repair the damage. Translesion synthesis (TLS), which promotes the completion of DNA replication instead of repairing damaged DNA, is one of the processes used by the cell to bypass damage during S phase. This process is initiated by the monoubiquitylation of PCNA, which subsequently triggers the recruitment of specialized DNA polymerases to the replication fork (Friedberg et al., 2002). These specialized polymerases are Polζ (also referred to as Rev3), a member of the B family of DNA polymerases, and members of the Y family of DNA polymerases; the latter group consists of Rev1, Polι (POLI), Polη (POLH), and Polκ (POLK) (reviewed by Shaheen et al., 2010; Lehmann, 2006).

The Y family of DNA polymerases is conserved from bacteria to humans. Ultraviolet light (UV) is a major trigger to recruit these polymerases to stalled replication forks (Waters et al., 2009; Jarosz et al., 2007; Lehmann, 2006). Mutation of one of the human Y-family polymerases, POLH, results in a variant form of Xeroderma Pigmentosum (XP-V), a disease characterized by UV sensitivity and a high susceptibility to skin cancers (Masutani et al., 1999; Kannouche and Stary, 2003; Kannouche et al., 2001). *polh*$^{-/-}$ null mice also exhibit high mutation frequencies and UV-induced epithelial tumors (Busuttil et al., 2008; Ohkumo et al., 2006).

Drosophila has three members of the Y family of DNA polymerases: DNApol-eta, DNApol-iota and Rev1. *Drosophila* DNApol-eta and DNApol-iota are functional

translesion polymerases *in vitro* (Ishikawa et al., 2001), but there are no reported mutant alleles for the genes encoding these polymerases in flies. Knockdown of *polh-1*, the *C. elegans DNApol-eta* homolog, in the germ line results in increased sensitivity of early embryos to UV radiation (Ohkumo et al., 2006). Recent studies suggest that POLH-1 may play a key role during early *C. elegans* embryogenesis to keep the rapid cycles on schedule (Holway et al., 2006; Kim and Michael, 2008). Because the Y polymerases are conserved in eukaryotes and expressed during early development, it seems likely that Y-family polymerases play similar roles in cell-cycle progression during the development of other organisms.

We previously described a *Drosophila* maternal effect-lethal mutant that we named "*no poles*" (*nopo*) (Merkle et al., 2009). Embryos from *nopo* females undergo mitotic arrest during the rapid S-M cycles of syncytial embryogenesis. Checkpoint kinase 2 (Chk2) plays a unique checkpoint function in the *Drosophila* early embryo as it is locally activated in response to DNA damage or incomplete replication. We showed that Chk2 is activated in *nopo* mutants, suggesting that NOPO plays a role in preserving genomic integrity during early embryogenesis of *Drosophila*. We also showed that syncytial embryos lacking NOPO exhibit a significantly shorter interphase 11 as compared to wild type, suggesting that *nopo* mutants may enter mitosis prior to the completion of DNA replication, thereby triggering Chk2 activation.

nopo, which encodes a candidate RING domain-containing E3 ubiquitin ligase, is the *Drosophila* homolog of a 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 result in cell proliferation, activation,

differentiation, and apoptosis (reviewed by Ha et al., 2009); however TRIP's physiological role in TNF signaling is unclear (Lee et al., 1997; Regamey et al., 2003). Although substrates of mammalian TRIP have not been reported to date, the mouse TRIP homolog is a functional E3 ligase *in vitro* (Besse et al., 2007).

Ubiquitylation is a posttranslational modification that plays an important role in many cellular processes, including protein processing, cell cycle control, chromatin remodeling, DNA repair, and membrane trafficking (Liu and Chen, 2011; Broemer and Meier, 2009; Le Bras et al., 2011; Acconcia et al., 2009; Hershko, 1997; Al-Hakim et al., 2010; O'Connell and Harper, 2007). Modification of proteins by ubiquitylation is a multi-step process that involves a ubiquitin activating enzyme (E1), ubiquitin conjugating enzymes (E2s or Ubcs), ubiquitin ligases (E3s), and ubiquitin (Ub) (reviewed by Fang and Weissman, 2004; Weissman, 2001; Wilkinson, 2000). Ubiquitylation involves the linkage of one or more ubiquitin molecules to another protein; the resulting modification may alter its fate in a number of ways, including the following: targeting it for destruction by the 26S proteasome, changing its subcellular location, or changing its protein-protein interactions (Glickman and Ciechanover, 2002).

To identify potential ubiquitylation substrates of the E3 ligases NOPO and TRIP and to elucidate the mechanism by which NOPO/TRIP promotes genomic stability, we performed a yeast two-hybrid screen using human TRIP. We report here the interaction of *Drosophila* NOPO and mammalian TRIP with members of the Y family of DNA polymerases. We show that the E3 ligases NOPO and TRIP enhance the ubiquitylation of the Y polymerase proteins, suggesting that NOPO plays an important role in regulating

the Y-family polymerases during the rapid syncytial cell cycles of *Drosophila* embryogenesis.

RESULTS

Drosophila NOPO and mammalian TRIP are functional homologs

We previously described a *Drosophila* maternal effect-lethal mutant that we named "*no poles*" ("*nopo"*) (Merkle et al., 2009). *nopo*, which encodes a candidate RING domain-containing E3 ubiquitin ligase, is the *Drosophila* homolog of a human gene encoding TRAF-interacting protein (hTRIP). To test functional conservation of the human and *Drosophila* homologs, we investigated whether transgenic expression of human *hTRIP* could rescue fly *nopo* mutants. Embryos derived from *nopo* females undergo mitotic arrest with barrel-shaped, acentrosomal spindles during the rapid S-M cycles of syncytial embryogenesis (Fig. 4.1C,D). We first tested whether these defects of *nopo* mutants could be rescued by expressing *nopo* cDNA under the control of the endogenous *nopo* promoter (*nopo* cDNA rescue, Fig. 4.1A). We found that this transgene restored fertility to *nopo* females: whereas embryos from control *nopo* females never hatched into larvae, embryos produced by *nopo* females carrying the *nopo* cDNA rescue transgene exhibited a 67% hatch rate compared to 82% in wild type (Fig. 4.1B). To test where NOPO and hTRIP are functionally conserved, we similarly expressed human *hTRIP* under the control of the endogenous *nopo* promoter (*hTRIP* cDNA rescue) (Fig. 4.1A). Fertility was restored in *nopo* mutant females carrying the *hTRIP* cDNA rescue transgene, resulting in a 59% hatch rate (Fig. 4.1B). Mitotic spindles were also restored to

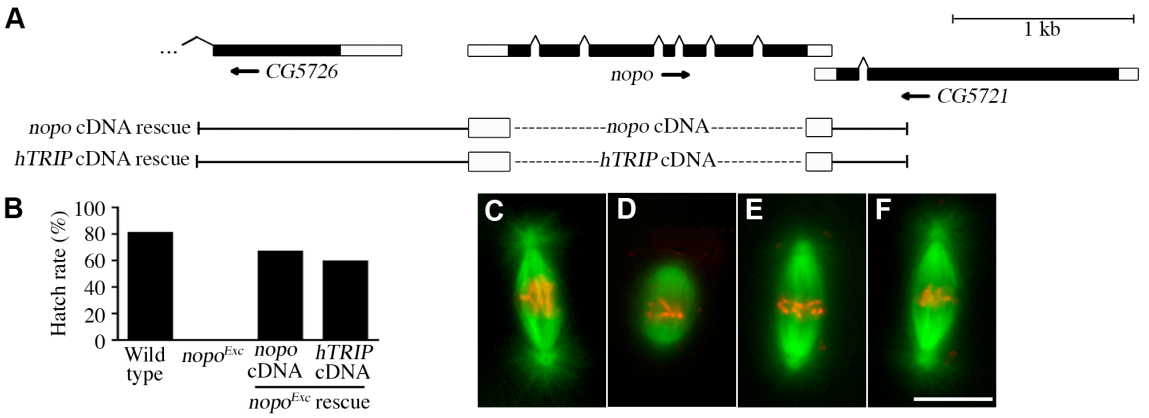


Figure 4.1: Human TRIP is the functional homolog of *Drosophila* NOPO. (A) *nopo* genomic region (top). Coding regions are represented by black boxes, 5'- and 3'-UTRs by white boxes, spliced regions by lines. Arrows indicate transcription direction. Transgenic rescue constructs (bottom). Rescue constructs contained genomic sequences flanking *nopo* (lines), 5'- and 3'-UTRs of *nopo* (white boxes), and coding regions of *nopo* or *hTRIP* cDNA (dashed lines). (B) The sterility of $nopo^{Exc}$ mutant females is almost completely rescued by transgenic expression of either *nopo* or *hTRIP* cDNA (constructs shown in A). (C-F) Representative mitotic spindles in syncytial embryos from females of the following genotypes: wild-type (C), *nopo* (D), or *nopo* with transgenic expression of NOPO (E) or hTRIP (F). Microtubules are in green and DNA in red. Bar, 10 μm.

a wild-type morphology in embryos from *nopo* females expressing either the *nopo* or *hTRIP* cDNA rescue transgenes (Fig. 4.1E,F). Thus, *Drosophila* NOPO and human TRIP are functionally conserved.

TRIP interacts with Y-family DNA polymerases via yeast two-hybrid assays

RING-containing E3 ligases often interact directly with their substrates (Deshaies and Joazeiro, 2009). To further understand NOPO's role in maintaining genomic integrity during early embryonic development in *Drosophila*, we sought to identify potential substrate(s) of NOPO 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 using the human homolog of NOPO, TRIP, as the bait and a HeLa cell cDNA library as the prey. Human TRIP cDNA was cloned into pGBKT7 vector (Matchmaker III from Clontech) and transformed into yeast, and expression of TRIP was verified by Western blot analysis (data not shown). Using yeast expressing the pGBKT7-hTRIP plasmid as the bait strain, we screened yeast pretransformed with a HeLa cell cDNA library for two-hybrid interactions (see Methods chapter for details). Sequence analysis revealed that one of the first clones to appear on selective media encoded human POLK, a member of the Y family of DNA polymerases.

To determine if TRIP interacts with other members of the Y family of DNA polymerases, which includes POLH, POLI, POLH and REV1, we individually tested each of these polymerases for interaction with TRIP via yeast two-hybrid assay. We cloned cDNAs encoding full-length and truncated versions of these polymerases into pGADT7 and transformed the prey plasmids into yeast. We found that all four family

members interact with TRIP (Fig. 4.2A-E). While TRIP exclusively interacts with the C-terminal half of POLH and POLK, TRIP interacts with both N- and C-terminal halves of POLI (Fig. 4.2A-C). These data are consistent with the many known protein-protein interaction domains located within the C-terminal region of the Y polymerases (Waters et al., 2009).

We also investigated the region of TRIP that is responsible for its interaction with the Y polymerases by generating truncated forms of TRIP bait that were transformed in yeast cells. When testing the capacity of the full-length Y polymerase prey proteins to interact with these truncated TRIP forms, we found that all four Y polymerases interact strongly with the N-terminal half of TRIP and only weakly with the C-terminal half of TRIP (Fig. 4.2D,E). These data suggest that TRIP binds the Y polymerases near its RING domain (Merkle et al., 2009).

NOPO interacts with Y-family DNA polymerases via yeast two-hybrid assays

The Y polymerases conserved in *Drosophila* are DNA-pol-eta, DNApol-iota and dREV1 (Fig. 4.6A). To determine if NOPO similarly interacts with the *Drosophila* Y polymerases, we generated yeast expressing pGBKT7-NOPO bait plasmid as well as yeast expressing cDNAs encoding full-length or truncated versions of *Drosophila* Y polymerases in the pGADT7 prey plasmid. We found that NOPO interacts with all *Drosophila* Y-family polymerase homologs (Fig 4.2F-I). To map the protein region responsible for the Y polymerase-NOPO interaction, we generated truncated forms of DNApol-eta and DNApol-iota and tested the capacity of these prey constructs to interact with full-length NOPO. We found that the C-terminal halves of DNApol-eta and

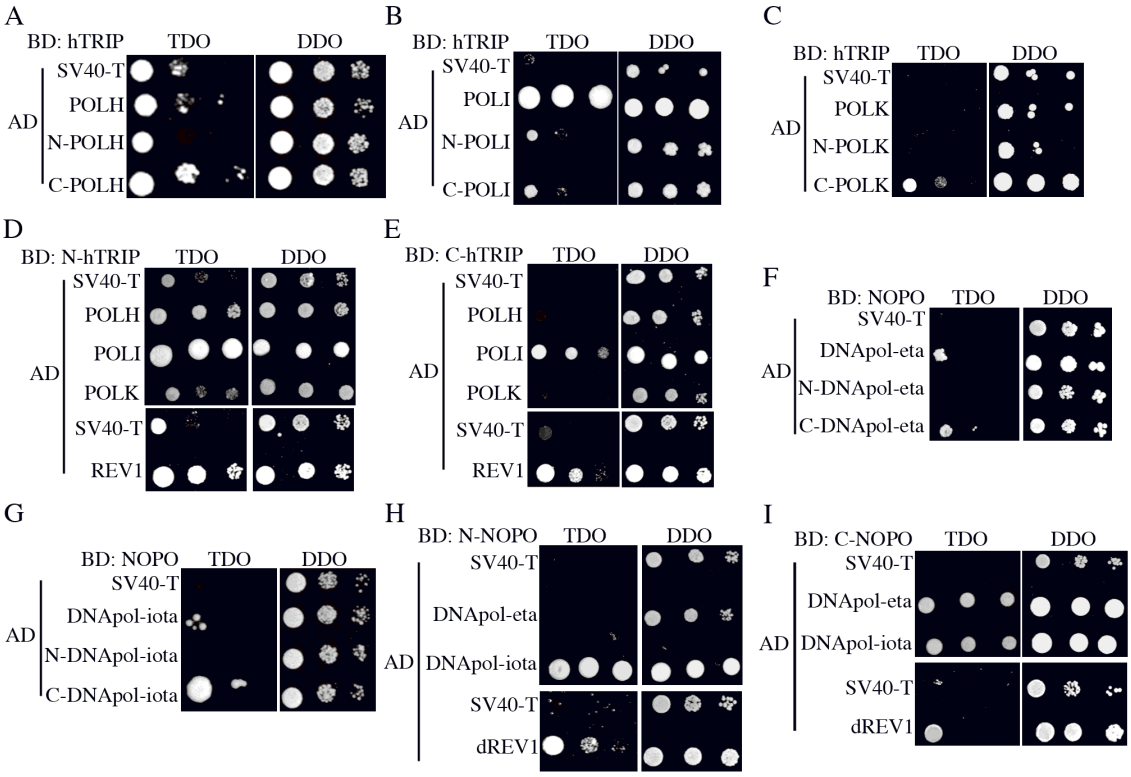


Figure 4.2: TRIP and NOPO interact with Y-family DNA polymerases in yeast two-hybrid assays. (A-I) Yeast cells expressing various baits fused to the DNA-binding domain of Gal4 (BD, "bait") and full-length or truncations of members of the indicated Y family of DNA polymerases fused to the Gal4 activation activation domain (AD, "prey") were spotted onto selective media. Physical interactions were tested by spotting serially diluted cells onto triple dropout (TDO) medium *(-Trp, -Leu, -His*) and scoring growth after 3 days at 30°C. Side by side plating on double dropout (DDO) media (*-Trp, -Leu*) was performed as a control. SV40 T-antigen (SV40-T) was used as a negative control prey for all bait constructs. (A-C) Full-length human TRIP was used as bait. TRIP interacts with full-length and/or the C-terminal ends of human Y-family DNA polymerases POLH (A), POLI (B), and POLK (C). (D,E) Truncations of TRIP were used as bait. (D) POLH, POLI, POLK, and REV1 interact with the N-terminal end of TRIP. (E) POLI and REV1 also interact with the C-terminal end of TRIP. (F,G) Full-length NOPO was used as bait. NOPO interacts with full-length and the C-terminal ends of DNApol-eta (F) and DNApol-iota (G). (H,I) Truncations of NOPO were used as bait. (H) DNApol-iota and dREV1 interact with the N-terminal end of NOPO. (I) DNApol-eta, DNApol-iota, and dREV1 interact with the C-terminal end of NOPO.

DNApol-iota are sufficient for interaction with NOPO (Fig. 4.2F,G). These data are consistent with our observations that the human Y polymerase proteins similarly interact with TRIP via their C-termini (Fig. 4.2A-C). Furthermore, these data fit well with the many protein-protein interaction domains previously identified within the C-terminal end of the *Drosophila* Y polymerase proteins (Waters et al., 2009).

To define the region of NOPO that interacts with the *Drosophila* Y polymerases, we transformed yeast cells with truncated forms of NOPO bait and tested their capacity to interact with full-length Y polymerase prey proteins. We found that DNApol-eta interacts exclusively with the C-terminal half of NOPO, whereas DNApol-iota and dREV1 interact with both N- and C-terminal halves of NOPO (Fig. 4.2H,I).

TRIP/NOPO E3 ligases interact with Y-family polymerases in cultured cells

To determine whether these interactions also occur in cultured cells, we co-expressed MYC-tagged human TRIP and epitope-tagged human Y polymerases in HeLa cells. We found that POLH, POLI and POLK could each be co-immunoprecipitated with TRIP (Fig. 4.3A-C). Similar results were obtained by immunoprecipitating each of the Y polymerases and immunoblotting for TRIP (data not shown). We similarly investigated the interaction of *Drosophila* Y polymerases with NOPO in cultured *Drosophila* S2 cells. We co-expressed MYC-tagged NOPO and HA-tagged *Drosophila* Y polymerases in S2 cells and found that DNApol-eta and DNApol-iota co-immunoprecipitated with NOPO (Fig. 4.3D,E). These data indicate that human and *Drosophila* Y polymerases can interact with TRIP and NOPO, respectively, in cultured cells. Interestingly, we often observe a faint slower migrating band of the Y polymerases on immunoblots of cell lysates. These

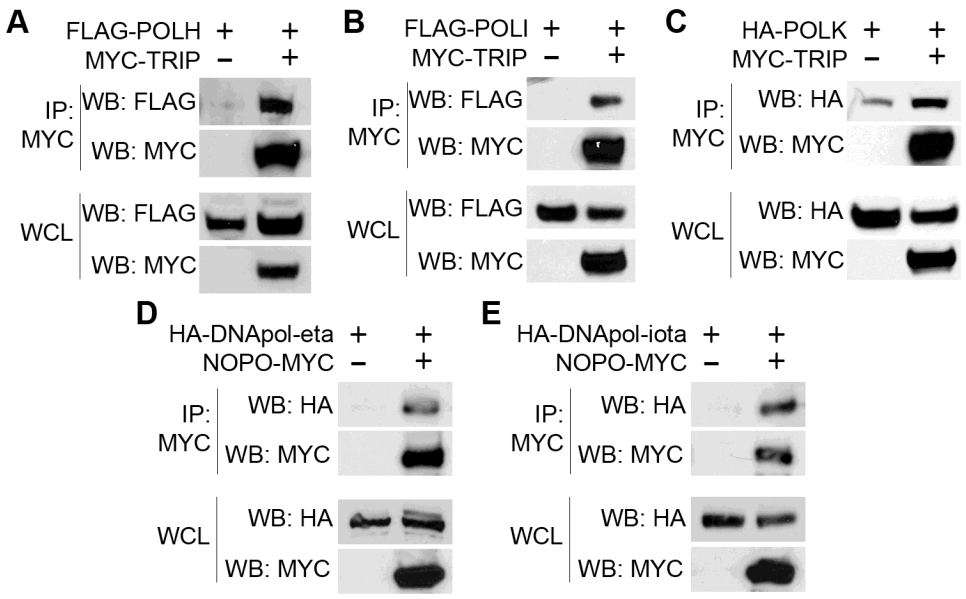


Figure 4.3: TRIP and NOPO interact with Y-family DNA polymerases in cultured cells. (A-C) HeLa cells were co-transfected with expression constructs encoding MYC-tagged TRIP or empty vector plus tagged versions of human Y-family polymerases as indicated. MYC-TRIP complexes were immunoprecipitated (IP) from cell lysates and resolved by SDS-PAGE/immunoblotting. The Y-family polymerases FLAG-POLH (A), FLAG-POLI (B), and HA-POLK (C) co-immunoprecipitated with MYC-TRIP. (D-E) *Drosophila* S2 cells were co-transfected with MYC-tagged NOPO or empty vector plus the indicated *Drosophila* Y-family polymerases. NOPO-MYC complexes were immunoprecipitated from cell lysates and resolved by SDS-PAGE/immunoblotting. HA-DNApol-eta (D) and HA-DNApol-iota (E) co-immunoprecipitated with NOPO-MYC. Immunoblotting of whole cell lysates (WCL) used in all IP assays is shown.

bands may represent monoubiquitylated forms of the Y polymerases, as reported previously (Bienko et al., 2010).

TRIP/NOPO E3 ligases enhance the ubiquitylation of Y-family polymerases in cultured cells

To determine whether the interactions between TRIP/NOPO and the Y polymerases is that of E3 ligases and their substrates, we performed ubiquitylation assays in cultured cells. To do this, we first tested whether HA-tagged Y polymerases POLH, POLI and POLK would become covalently linked to polyhistidine-tagged ubiquitin (His_6-Ub) in transiently transfected HeLa cells in the presence of MYC-tagged TRIP. As a control, we compared ubiquitylation of the HA-tagged Y polymerases in the absence of MYC-TRIP. As expected, HA-POLH and HA-POLK are monoubiquitylated in the absence of MYC-TRIP (Fig. 4.4A,B). These data confirm previous studies suggesting that the mammalian Y polymerases are monoubiquitylated (Bienko et al., 2010). In the presence of MYC-TRIP, ubiquitylation of HA-POLH and HA-POLK is enhanced, as indicated by the upward smearing on immunoblots of HA-labeled protein in cells co-expressing His_6-Ub, HA-tagged Y polymerase, and MYC-TRIP. The monoubiquitylation band and the ubiquitin smear were only observed in the presence of His_6-Ub, consistent with covalent linkages of His-Ub to the Y polymerases. Unlike POLH and POLK, epitope-tagged POLI did not show a significant enhancement of its ubiquitylation in the presence of MYC-TRIP in this assay (data not shown). These data show that TRIP can promote polyubiquitylation of the Y polymerases POLH and POLK in cultured mammalian cells.

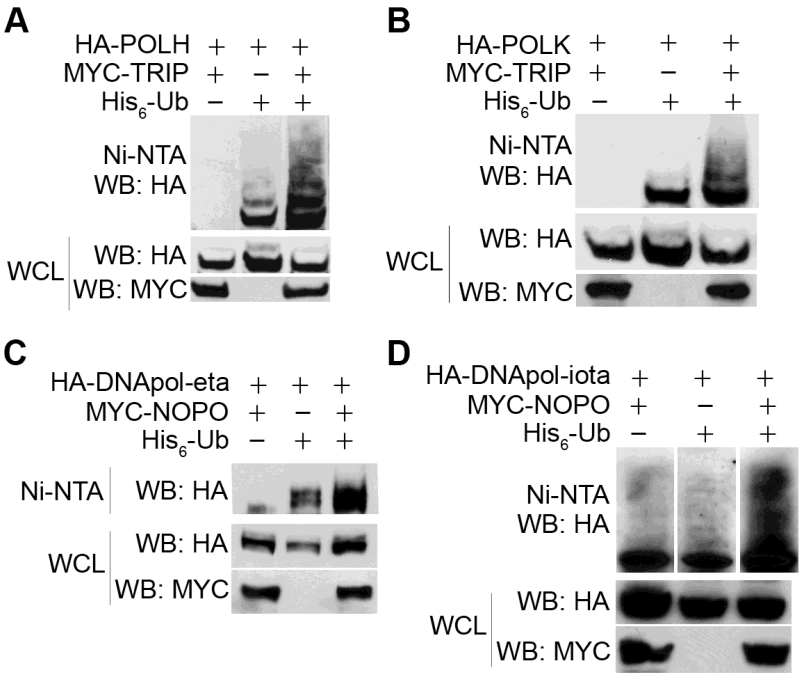


Figure 4.4: TRIP and NOPO enhance the ubiquitylation of Y-family polymerases. (A,B) HeLa cells were co-transfected with expression constructs encoding MYC-TRIP, HA-tagged versions of human Y-family DNA polymerases, and polyhistidine-tagged Ub (His_6-Ub) as indicated. After denaturing cell lysis, nickel affinity chromatography was used to recover ubiquitylated proteins, which were resolved by SDS–PAGE/immunoblotting. MYC-hTRIP enhanced the ubiquitylation of HA-POLH (A) and HA-POLK (B). (C,D) *Drosophila* S2 cells were co-transfected with expression constructs encoding NOPO-MYC, HA-tagged versions of *Drosophila* Y-family DNA polymerases, and His_6-Ub as indicated. Analysis of ubiquitylated proteins was performed as in (A,B). NOPO-MYC enhanced the ubiquitylation of HA-DNApol-eta (C) and HA-DNApol-iota (D). Immunoblotting of whole cell lysates (WCL) used in all ubiquitylation assays is shown. Intervening lanes were removed in panel D.

We similarly investigated the ubiquitylation of *Drosophila* Y polymerases by NOPO in cultured *Drosophila* S2 cells using a construct we generated to induce the expression of His_6-Ub. As in mammalian cells, we compared the ubiquitylation of HA-tagged DNApol-eta and DNApol-iota in the presence or absence of MYC-NOPO (Fig. 4.4C,D). HA-DNApol-eta and HA-DNApol-iota appear to be monoubiquitylated when MYC-NOPO is not co-expressed, suggesting that *Drosophila* Y polymerases normally undergo this modification. Although this modification has been shown in budding yeast, *C. elegans* and mammalian cells, ubiquitylation of the *Drosophila* Y polymerases has not been previously reported (Skoneczna et al., 2007; Kim and Michael, 2008; Jung et al., 2010; Bienko et al., 2010). We observed similar results as we did for the human homologs in the presence of the E3 ligase. With co-expression of MYC-NOPO, ubiquitylation of HA-DNApol-eta and HA-DNApol-iota is enhanced, as indicated by the upward smearing of HA-labeled protein on immunoblots. Again, the monoubiquitylation band and the ubiquitin smear were only observed in the presence of His_6-Ub. These data show that NOPO can polyubiquitylate the Y polymerases DNApol-eta and DNApol-iota in *Drosophila* cultured cells.

POLH co-localizes with TRIP in cultured human cells

We previously reported that N-terminally tagged eGFP-TRIP localizes to nuclear puncta during G2 phase in transiently transfected HeLa cells (Merkle et al., 2009). Another group reported that endogenous TRIP localizes to the nucleolus in MCF7(BD) breast epithelial cells (Zhou and Geahlen, 2009). We confirmed their results by transiently transfecting C-terminally tagged TRIP-mCherry in HeLa cells and co-

localizing TRIP with a nucleolar marker; however, we also observe TRIP-mCherry localized to nuclear puncta in transfected human cells (data not shown).

To obtain further evidence that TRIP and the Y-family polymerases interact, we compared the localization patterns of fluorescently tagged versions of these proteins in transfected HeLa cells. When transfected alone in the absence of UV, eGFP-POLH localizes to the nucleus and seldomly to nuclear puncta (Fig. 4.5A). In a low percentage of cells, a small pool of eGFP-POLH was detected in the nucleolus; in the majority of cells, however, eGFP-POLH is nuclear and does not localize to the nucleolus, as shown immuostaining with the nucleolar marker anti-Fibrillarin (Fig. 4.5A). After treatment with UV, eGFP-POLH localizes to nuclear puncta, which indicates its recruitment to stalled replication forks during translesion synthesis (Kannouche et al., 2003; Kannouche and Lehmann, 2006). When eGFP-POLH and TRIP-mCherry were co-transfected in untreated cells, however, eGFP-POLH localized to the nucleolus of cells positive for TRIP-mCherry nuclear puncta (Fig. 4.5B). After treatment with UV, eGFP-POLH was localized both to nuclear puncta and co-localized with TRIP-mCherry to the nucleolus. These data suggest that TRIP can recruit Y-family polymerases to the nucleolus and provide further evidence for *in vivo* interactions between these proteins.

Drosophila Y-family polymerases are expressed during early embryogenesis

We were interested in the potential role of Y-family polymerases as NOPO interactors/substrates during early embryogenesis of *Drosophila* and whether their regulation is a key mechanism by which NOPO promotes cell-cycle progression and preservation of genomic integrity. The Y polymerases conserved in *Drosophila* are

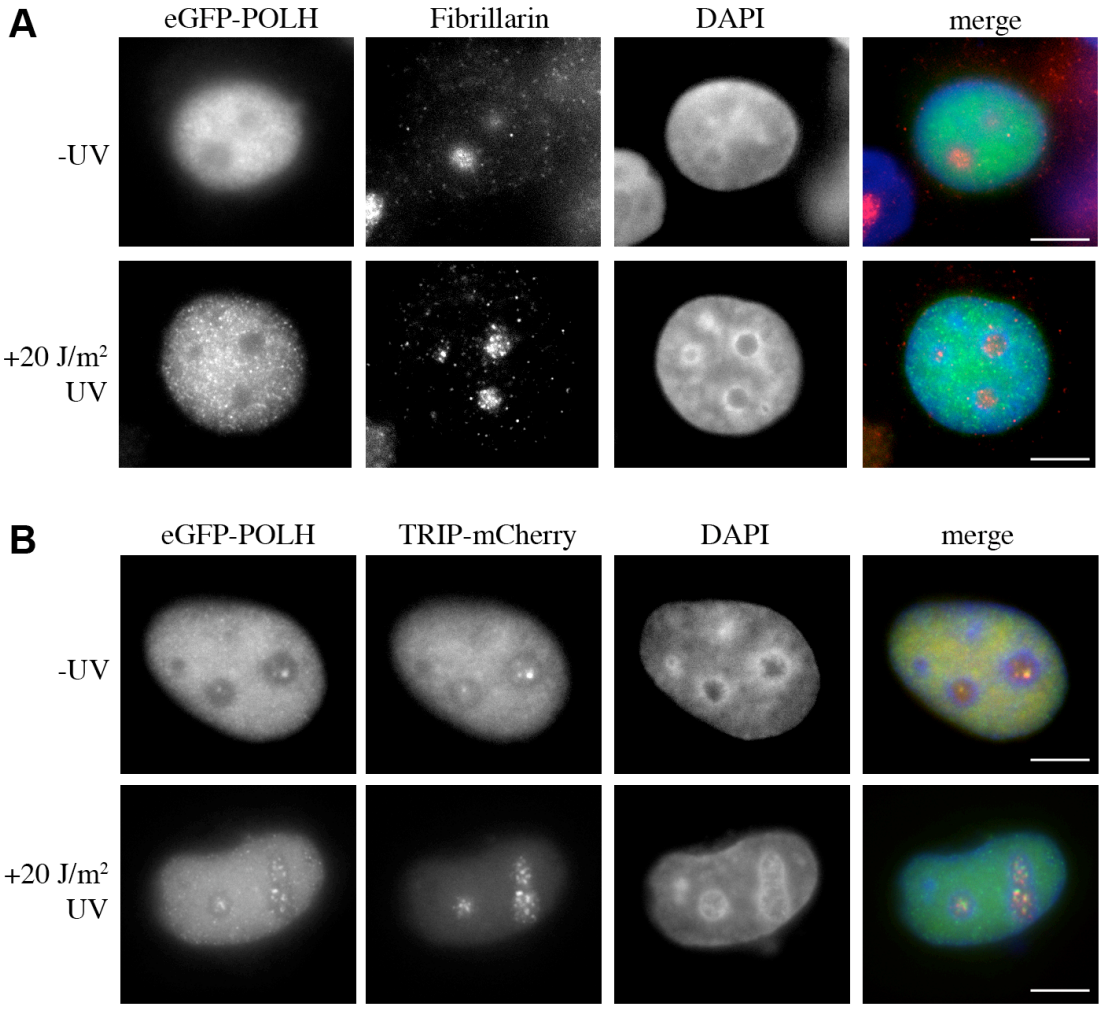


Figure 4.5: Localization of Y-family polymerases is altered by co-expression with TRIP in cultured mammalian cells. Immunofluorescent micrograms of transfected HeLa cells. DNA is in blue. (A) eGFP-POLH (green) is diffusely nuclear in untreated cells and localizes to small nuclear puncta after UV treatment (20 J/m^2). With or without UV treatment, eGFP-POLH generally does not co-localize with the nucleolar marker anti-Fibrillarin (red). (B) Co-transfection of eGFP-POLH (green) with TRIP-mCherry (red) promotes its localization to the nucleolus both in untreated and UV-treated cells (merge). Bars, 10 μm.

DNA-pol-eta, DNApol-iota and dREV1; a POLK homolog does not appear to be present in *Drosophila* (Fig. 4.6A). To determine if these polymerases are expressed in early *Drosophila* embryogenesis, we performed reverse transcriptase (RT-) PCR on mRNA extracted from 0-2 hour *Drosophila* embryos. We found that all of the Y polymerase genes are co-expressed with *nopo* during syncytial embryogenesis (Fig. 4.6B). These results suggest that, as in *C. elegans*, the *Drosophila* Y polymerases may have a unique role during the maternally-controlled S-M cell cycles of early embryogenesis.

DNApol-eta females have reduced fertility and produce embryos with _nopo_-like defects

Mutant alleles have not been reported for any of the *Drosophila* Y-family polymerases. We therefore generated a null allele of *DNApol-eta* (*Exc176*) via imprecise excision of *P*-element *EY07711* inserted in the 5'-UTR of the *DNApol-eta* gene region (Fig. 4.6C; see Chapter 5 for methods). Homozygous *DNApol-eta*^{Exc176} adults are viable, although they appear to have decreased longevity compared to wild type (data not shown). *DNApol-eta*^{Exc176} adult males display wild-type fertility, but homozygous and hemizygous mutant females have decreased fertility (~50% of wild type; Fig. 4.6D). This reduction in fertility is partially rescued by a transgene expressing HA-tagged DNApol-eta. Immunostaining of *DNApol-eta*^{Exc176}-derived embryos revealed that they arrest early in syncytial development with mitotic spindle defects strikingly reminiscent of those observed in embryos from *nopo* females (Fig. 4.6E,F). Embryos of *DNApol-eta*^{Exc176} homozygotes are often (41%) acentrosomal, barrel-shaped, variable in width, and have

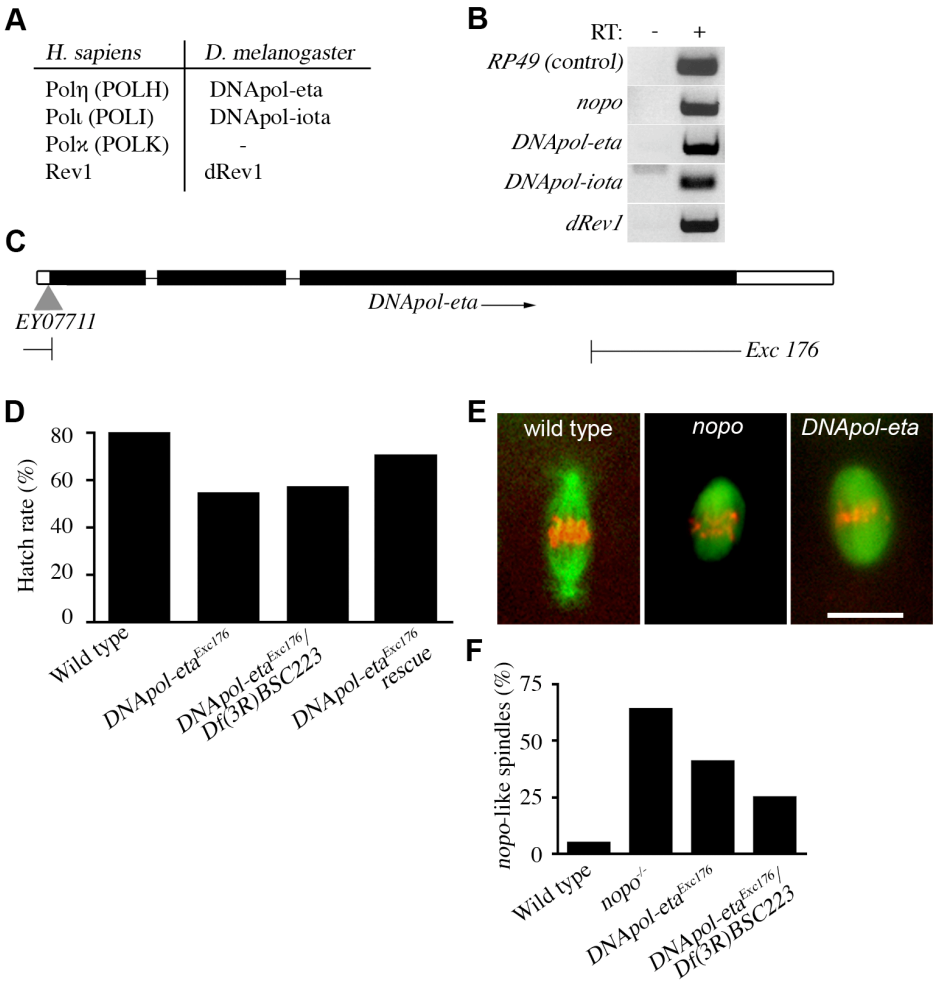


Figure 4.6: *DNApol-eta* mutants phenocopy *nopo* mutants. (A) Conservation of Y-family polymerases in *Homo sapiens* and *Drosophila melanogaster*. (B) *nopo* and the *Drosophila* Y polymerases *DNApol-eta*, *DNApol-iota*, and *dRev1* are expressed in 0-2 hour *Drosophila* embryos. Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed on mRNA derived from syncytial embryos followed by agarose gel electrophoresis. Reactions lacking reverse transcriptase (-RT) are shown as controls. (C) *DNApol-eta* gene structure. Coding regions are represented by black boxes, 5'- and 3'-UTRs by white boxes, and spliced regions by lines. Arrow indicates transcription direction. Imprecise excision of *P*-element *EY07711* (indicated by triangle) generated *DNApol-eta*^{Exc176} (gap represents deleted region). (D) Fertility (determined by hatch rates) of *DNApol-eta*^{Exc176} and *DNApol-eta*^{Exc176}*/Df(3R)BSC223* females compared to wild type. The decreased fertility of *DNApol-eta*^{Exc176} females is partially rescued by a transgene expressing HA-tagged *DNApol-eta* cDNA. (E) Representative mitotic spindles in embryos from wild-type, *nopo, or DNApol-eta*^{Exc176} females. Staining of *nopo*-derived and *DNApol-eta*^{Exc176}-derived embryos reveals shortened, barrel-shaped spindles with detached centrosomes that differ from wild type. Microtubules are in green and DNA in red. Bars, 10 μm. (F) Quantification of *nopo*-like barrel-shaped spindles with detached centrosomes in *nopo*-derived and *DNApol-eta*^{Exc176}-derived embryos.

misaligned chromosomes; similar phenotypes were observed in embryos of *DNApol-eta*^{Exc176} hemizygotes (25%) (Fig. 4.6E,F).

DNApol-eta localizes to interphase nuclei in early embryos of *Drosophila*

To further investigate the function of DNApol-eta in the early embryo, we visualized HA-tagged DNApol-eta protein in transgenic flies during the early syncytial divisions and found it to be exclusively localized to interphase nuclei (Fig. 4.7A). This localization is consistent with its reported nuclear localization in cultured cells (as in Fig. 4.5A; Kannouche et al., 2003; Kannouche and Lehmann, 2006).

DNApol-eta interacts with NOPO in early embryos of *Drosophila*

Since *DNApol-eta* mRNA is present in the early embryo, we tested whether DNApol-eta protein physically interacts with NOPO during this stage of *Drosophila* development. We expressed HA-DNApol-eta in the female germ line of wild-type and *nopo* null females (as negative control) followed by immunoprecipitation of endogenous NOPO. We found that DNApol-eta co-immunoprecipitated with NOPO from wild-type embryos (Fig. 4.7B). Interestingly, we typically observe a faint, slower migrating band of HA-DNApol-eta on immunoblots of early embryonic lysates. This upward-shifted band may represent a monoubiquitylated form of HA-DNApol-eta, as observed in lysates of cultured mammalian and *Drosophila* cells (as in Figs. 4.3,4.4). Taken together, the yeast two-hybrid interactions, co-immunoprecipitation, and similar mutant phenotypes that we observe suggest that NOPO and DNApol-eta function in a common pathway to preserve genomic integrity during early embryonic development in *Drosophila*.

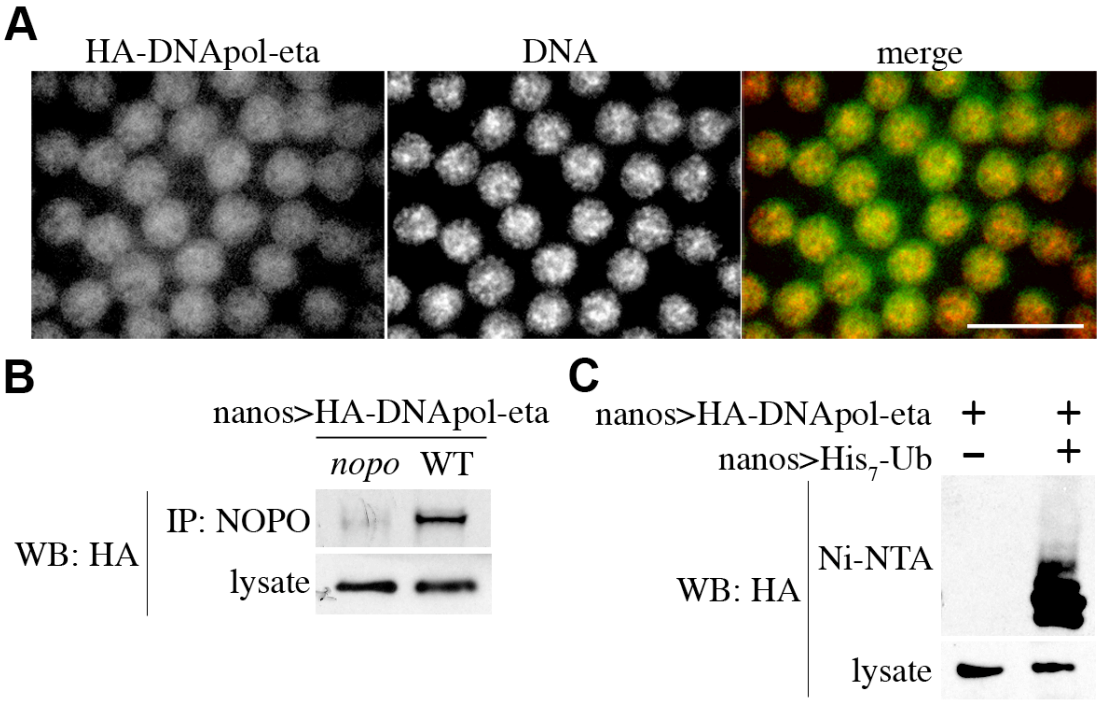


Figure 4.7: DNApol-eta interacts with NOPO and is ubiquitylated in the early *Drosophila* embryo. (A) HA-DNApol-eta is a nuclear protein in the early *Drosophila* embryo. Expression of HA-tagged DNApol-eta was driven by nanos-Gal4 in the female germline and syncytial embryos from these females were fixed and immunostained for the HA-epitope tag (green) and DNA (red). HA-DNApol-eta is exclusively localized to interphase nuclei. Bars, 20 μm. (B) HA-DNApol-eta coimmunoprecipitates with NOPO in the early *Drosophila* embryo. Immunoprecipitation (IP) of endogenous NOPO from syncytial embryos derived from wild type or *nopo* females expressing HA-DNApol-eta driven by nanos-Gal4 in the female germline. IP was performed on embryo lysates to recover proteins that interact with NOPO, which were resolved by SDS-PAGE and immunoblotting. (C) DNApol-eta is ubiquitylated in the early *Drosophila* embryo. Embryos expressing HA-DNApol-eta, in the presence or absence of His_7-ubiquitin, was driven by nanos-Gal4 in the female germ line of wild type females. Syncytial embryos derived from females expressing nanos>His_7-ubiquitin, HA-DNApol-eta or nanos>HA-DNApol-eta were lysed under denaturing conditions and nickel affinity chromatography was used to recover ubiquitylated proteins, which were resolved by SDS–PAGE and visualized by immunoblotting.

DNApol-eta is ubiquitylated in early embryos of *Drosophila*

To determine whether the interaction between *Drosophila* NOPO and DNApol-eta is that of an E3 ligase and its substrate, we performed an *in vivo* ubiquitylation assay using early embryos. To do this, we first tested whether HA-DNApol-eta could become covalently linked to polyhistidine-tagged ubiquitin (His_7-Ub) in syncytial embryos with transgenic expression of HA-DNApol-eta and His_7-Ub (Fig. 4.7C). Using a protocol similar to the one we used for HeLa cells, we found that HA-DNApol-eta can be polyubiquitylated in early embryos.

DISCUSSION

Our yeast two-hybrid screen identified members of the Y family of DNA polymerases as TRIP interactors. Subsequent work in cultured mammalian cells showed that TRIP interacts with and enhances the ubiquitylation of Y polymerases. We also found that the *Drosophila* Y polymerases are NOPO interactors. Because there were no reported mutant alleles for the Y polymerases in *Drosophila*, we generated a null mutation in *DNApol-eta* and observed decreased hatch rates and *nopo*-like spindle defects in embryos from *DNApol-eta* females. Together, these data suggest that *Drosophila* DNApol-eta has a unique role during early embryogenesis to promote cell-cycle progression and that NOPO regulates its activity. We propose a model in which the RING-containing E3 ubiquitin ligases NOPO/TRIP interact with and ubiquitylate Y polymerases in cultured cells and during syncytial embryogenesis of *Drosophila* to promote genomic integrity, cell-cycle progression, and continuation of development. Our previous studies showed that mutation of *nopo* results in truncation of S-phase and/or

spontaneous DNA damage (Merkle et al., 2009). Subsequent mitotic entry results in activation of Chk2, which leads to changes in spindle morphology, mitotic arrest, and failure of *nopo*-derived embryos to develop to cellularization. These data led us to propose a model in which NOPO regulates timing of S-M transitions in syncytial embryos to ensure that S-phase is of sufficient length to allow completion of DNA replication prior to mitotic entry.

In *C. elegans*, knockdown of the *DNApol-eta* homolog, *polh-1*, in the germ line results in increased sensitivity of early embryos to UV radiation (Ohkumo et al., 2006). This suggests that POLH-1, and perhaps TLS, play important roles in maintaining the genome during early embryogenesis. POLH-1 has been further proposed to prevent stalling of replication forks during the early embryonic cell cycles by quickly responding to and bypassing lesions (Holway et al., 2006; Kim and Michael, 2008). The authors proposed that POLH-1 takes the place of replicative polymerases during S-phase (via a polymerase-switching event), thereby keeping the rapid early embryonic cell cycles progressing on schedule. POLH-1 is regulated by sumoylation and ubiquitylation (Kim and Michael, 2008), suggesting that POLH-1 is protected from degradation by sumoylation, and ubiquitylation of POLH-1 presumably occurs once POLH-1 has successfully bypassed the lesion to prevent the polymerases from binding the chromatin. Although ubiquitylation or ubiquitin-mediated proteolysis of POLH-1 by the E3 ligase CRL4-Cdt2 has not specifically been shown, POLH-1 is stabilized when *cdt-2* is knocked down in the embryo. These data suggest a unique role for the Y-family polymerases during early embryonic cell cycles. The regulation of POLH-1 during *C. elegans* embryogenesis is important to keep the rapid cycles on schedule, as well as to keep them

away from the chromatin when they are not needed. These data support a highly regulated polymerase-switching model of the replicative and Y-family polymerases during S-phase of developing embryos. Given that the Y polymerases are conserved in eukaryotes and expressed during early development, it seems likely that Y-family polymerases play roles in cell-cycle progression at this developmental stage in other organisms.

From studies in budding and fission yeast and mammalian cells, UV has been shown to be a major trigger to recruit Y-family polymerases to monoubiquitylated PCNA at sites of stalled replication forks (Watanabe et al., 2004; Lehmann, 2005). In unchallenged cells, mammalian Y-family polymerases are localized throughout the nucleus. In the presence of UV, however, the polymerases are recruited to stalled replication forks and appear as foci on the chromatin during S-phase. We show that when TRIP and POLH are co-expressed in cultured mammalian cells, they co-localize to the nucleolus, both in the absence and presence of UV. This suggests that TRIP may play a role in the recruitment of POLH to the nucleolus. The function of TRIP at the nucleolus is unknown; however, the recruitment of POLH away from nuclear foci in the presence of UV when TRIP is co-expressed is particularly striking. We propose that NOPO/TRIP-dependent ubiquitylation of the Y-family polymerases results in their sequestration to the nucleolus, thereby preventing the polymerases from being recruited to monoubiquitylated PCNA on the chromatin. We favor a model in which, in the absence of DNA damage, TRIP sequesters Y-family polymerases to the nucleolus so they cannot associate with the chromatin and interfere with the function of canonical DNA polymerases. When a lesion is detected, however, release of the Y-family polymerases from the nucleolus is triggered

to allow their interaction with monoubiquitylated PCNA on the chromatin and bypass of the DNA lesion.

TRAFs are key adaptor molecules in the TNF-signaling pathway that result in cell proliferation, activation, differentiation and apoptosis; however, TRIP's physiological role in TNF signaling is unclear (Lee et al., 1997; Regamey et al., 2003). Little has been reported about TRIP regulation, but a recent study showed that the tumor suppressor Syk phosphorylates TRIP in the presence of TNF and that overexpression of TRIP sensitizes cells to TNF-induced apoptosis (Zhou and Geahlen, 2009). Furthermore, when TRIP is knocked down in keratinocytes, cells exhibit an NF-κB-independent G1/S phase cell-cycle arrest (Almeida et al., 2011). It will be interesting to see if any of these proposed roles for TRIP in apoptosis and cell proliferation involve its regulation of Y polymerases.

There is also evidence that mammalian TRIP is critical during development. Homozygous mutant *TRIP* mouse embryos undergo an 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). These data show an upregulation of TRIP in the developing male anteroventral periventricular (AVPV) nucleus. The size difference in male versus female AVPV nuclei results from apoptosis in developing male neurons. Further investigation is likely to reveal new developmental roles for TRIP in TNF-dependent and –independent processes.

Despite efforts using a variety of approaches, we are not able to localize NOPO in the early embryo. Therefore, we cannot perform co-localization experiments between NOPO and Y-family polymerases. Using a transgenic line expressing HA-DNApol-eta, we show that HA-DNApol-eta is localized to the nucleus during S-phase of syncytial

embryogenesis. The localization of HA-DNApol-eta in the *nopo* mutant background was difficult to assess, however, as we found in preliminary experiments that these embryos arrested earlier than the control *nopo*-derived embryos. While follow-up experiments are needed, these data would be consistent with our model that NOPO plays a critical role in limiting the activity of DNApol-eta in the early embryo.

Further experimentation is required to determine if NOPO directly ubiquitylates the Y polymerases during early embryogenesis. A key experiment in addressing this question would be to perform *in vivo* ubiquitylation assays in early embryos null for *nopo*. Based on our observation that ubiquitylation of HA-DNApol-eta in S2 cells is enhanced when NOPO is co-expressed, we predict that HA-DNApol-eta ubiquitylation will be reduced or undetectable in *nopo*-null embryos. Such a result would more definitively point to a role for the NOPO E3 ligase in regulating Y-family polymerases during early embryogenesis of *Drosophila*. We do not see a detectable decrease in the levels of Y polymerases in the presence of NOPO/TRIP. Given that K63-linked ubiquitin chains generally act as non-proteolytic signals (Spence et al., 1995; Aguilar and Wendland, 2003), we propose that NOPO/TRIP E3s mediate assembly of K63-linked polyubiquitin chains on the Y polymerases to preserve genomic integrity in mammalian cells and in early embryogenesis of *Drosophila*.

We propose that *Drosophila* DNApol-eta has a unique role during early embryogenesis and that its activity and association with chromatin is regulated by NOPO. We favor a model in which NOPO ubiquitylates Y-family DNA polymerases during S-phase of early *Drosophila* embryogenesis and that ubiquitylation of these polymerases by NOPO regulates their localization and interaction with chromatin. More specifically, we

predict that NOPO-dependent ubiquitylation of the Y-family polymerases results in their sequestration away from the chromatin so that canonical polymerases can bind to the chromatin and promote high-fidelity DNA synthesis.

CHAPTER V

MATERIALS AND METHODS

DROSOPHILA STOCKS

Flies were maintained at 25˚C using standard techniques. *y w* was used as wild type unless otherwise indicated. *cn Z2-1447 bw/CyO* was a gift from Charles Zuker; *UASp-His$_7$-Ub* was a gift from Lynn Cooley; *ben^1* and *mnk^{6006}* stocks were from Mark Tanouye and Bill Theurkauf, respectively; and the *EYG5845* stock was from GenExel (Seoul, Korea). Other fly stocks were from Bloomington or Szeged stock centers.

QUANTIFICATION OF EGG HATCH RATES

Five newly eclosed females of the indicated genotype and five wild-type males were incubated in yeast-pasted vials for two days and transferred to egg-collection chambers at 25˚C. Eggs were collected daily over five days and scored for hatching ~40 hours post-collection (>500 eggs per genotype). Hatch rate is the ratio of hatched to total eggs expressed as a percentage.

GENETIC AND MOLECULAR MAPPING OF *nopo*

We screened a second chromosome deficiency collection for non-complementation of female sterility of *nopo^{Z1447}*. Females carrying *nopo^{Z1447}* in trans to any of several overlapping deficiencies (*Df(2R)Pcl-11B*, *Df(2R)Pcl-XM82*, *Df(2R)Pcl-7B*, or *Df(2R)PC4*) were sterile, placing *nopo* in the 55A1-C1 interval.

We further mapped *nopo*^{Z1447} by *P*-element-induced male recombination (Chen et al., 1998) relative to several insertions: *lolal*^{EP2169}, *Dgp-1*^{BG00396}, *CG5721*^{EY03388}, *fj*^{KG03419}, and *EP(2)1081*. Multiple independent recombinant chromosomes were recovered for each *P*-element tested. We narrowed *nopo* to five candidates in the 55B11-12 region (*Dgp-1*, *CG10916*, *CG5726*, *CG5140*, and *CG5721*) distal to *Dgp-1*^{BG00396} and proximal to *CG5721*^{EY03388} as annotated on FlyBase (Grumbling and Strelets, 2006).

For each candidate gene, coding regions were sequenced as described (Rickmyre et al, 2007). *nopo*^{Z1447} is a missense mutation in *CG5140* causing a glutamic acid to lysine change at residue 11 of the predicted protein. *Df(2R)Exel7153*, which deletes 15 genes in this region, was subsequently found to uncover *nopo*.

Putative *nopo* homologs were identified using HomoloGene (release 56), and NOPO's RING domain was identified using ScanProsite.

GENERATION OF *nopo*-NULL ALLELE

A *nopo*-null allele was generated by imprecise excision of *P*-element *EYG5845*. The 771-bp deletion *nopo*^{Exc142} lacks part of the 5'-UTR and exons encoding residues 1-181.

cDNA CLONES

cDNA encoding NOPO, BEN, UEV1A, DNApol-eta, DNApol-iota, dRev1, MAGO, and CG30291 (GH03577, LD24448, LD28904, SD05329, LD29090, GH11153, RE14116, and GH19637 respectively) were from the *Drosophila* Gene Collection. Human TRIP cDNA (ID 2821007) and MAGOH (ID 3861094) were from Open

Biosystems. Plasmids containing human POLH, POLI, POLK, and REV1 were a gift from Peter Guengerich. Plasmids containing human MDM2 and LZAP were gifts from Christine Eischen and Dell Yarbrough, respectively.

TRANSGENESIS

A 3.8 kb genomic fragment containing *CG5140* and flanking regions (Fig. 2.2A) was PCR-amplified from BAC clone BACR15G20 (*Drosophila* Genomics Resource Center) and subcloned into pCaSpeR4. A transgenic line carrying *pCaSpeR4-CG5140* was generated by *P*-element-mediated transformation via embryo injection (Rubin and Spradling, 1982).

For *nopo* cDNA and *hTRIP* cDNA rescue experiments *pCaSpeR4-CG5140* was used as a template for PCR in order to excise the *nopo* gene between the 5'- and 3'-UTRs. *nopo* or human *TRIP* cDNA was subsequently subcloned into the excised region (Fig. 3.1A). Transgenic lines carrying *pCaSpeR4-*$nopo_{gen}nopo_{cDNA}$ or *pCaSpeR4-*$nopo_{gen}hTRIP_{cDNA}$ were generated.

For *DNApol-eta* rescue and overexpression experiments, cDNA encoding *DNApol-eta* was PCR-amplified from clone SD05329 (*Drosophila* Genomics Resource Center) and subcloned into a HA-tagged version of UASp (Rørth, 1998). A transgenic line carrying *pUASp-HA-DNApol-eta* was generated.

EMBRYO IMMUNOSTAINING AND MICROSCOPY

Methods for fixation, staining, and fluorescence microscopy of embryos (1.5-2.5 hours unless otherwise indicated) and live-image analysis were previously described

(Rickmyre et al., 2007). *P*-values for live-image data were obtained using a two-tailed unpaired Student's *t*-test.

NOPO POLYCLONAL ANTIBODIES

A fusion consisting of an N-terminal MBP tag and C-terminal NOPO was used to generate anti-NOPO antibodies. DNA encoding C-terminal NOPO (residues 224 to 435) was PCR-amplified and subcloned into pMAL (New England Biolabs). MBP-C-NOPO was produced in bacteria, purified using amylose resin, and injected into guinea pigs (Covance).

PROTEIN EXTRACTS AND IMMUNOBLOTS

In Chapter 2, protein extracts were made by homogenizing embryos (1-2 hours) or dissected tissues in urea sample buffer (Tang et al., 1998). Proteins were transferred to nitrocellulose for immunoblotting using standard techniques. Antibodies were used as follows: guinea pig anti-NOPO (1:1000), mouse anti-GAPDH (1:1000, Abcam), mouse anti-α-tubulin (DM1α, 1:5000, Sigma), mouse anti-Cyclin B (F2F4, 1:200, Developmental Studies Hybridoma Bank), and rabbit anti-pY15-Cdk1 (1:1000, Upstate).

MAMMALIAN CELL TRANSFECTION, STAINING, AND MICROSCOPY

HeLa cells were maintained in Dulbecco's modified Eagle Medium (DMEM) containing 10% fetal bovine serum. Plasmids encoding N-terminally tagged (eGFP or mCherry) versions of NOPO, TRIP, and BEN generated by subcloning into pCS2 were

transfected into cells using Lipofectamine 2000 (Invitrogen) according to manufacturer's directions.

Cells were plated on fibronectin-coated coverslips 21 hours post-transfection and fixed three hours later. For direct fluorescence and centromere staining, cells were fixed 20 minutes with 4% formaldehyde in CBS (10 mM MES pH 6.1, 138 mM KCl, 3 mM $MgCl_2$, 2 mM EGTA, 0.32 M sucrose). For PCNA staining, cells were fixed 5 minutes in 70% methanol/30% acetone. For Cyclin A staining, cells were fixed 20 minutes in 3% paraformaldehyde/20% sucrose in phosphate-buffered saline. Cells were permeabilized 10 minutes with 0.5% Triton X-100 in Tris-buffered saline. Primary antibodies were used as follows: human autoimmune (CREST) serum (1:1000, ImmunoVision), Cyclin A (H-432, 1:100, Santa Cruz Biotechnology), and PCNA (PC10, 1:200 Santa Cruz Biotechnology). To visualize actin, cells were stained one hour with fluorescently conjugated phalloidin (1:1000, Invitrogen). Fluorescently conjugated secondary antibodies were used at 1:5000. Slides were mounted in Vectashield with DAPI (Vector Labs). Images were acquired using a Nikon Eclipse 80i microscope equipped with a CoolSNAP ES camera (Photometrics) and Plan-Apo 60X objective. For experiments involving quantification, at least 400 cells per condition were scored.

YEAST TWO-HYBRID ASSAYS

In Chapter 2, yeast two-hybrid assays were performed as described (James et al., 1996). Plasmids expressing wild-type and mutant versions of NOPO, BEN, and UEV1A fused to Gal4 DNA binding domain ("bait" vector pGBD-C) or Gal4 activation domain ("prey" vector pGAD-C) were transformed into *S. cerevisiae* strain PJ69-4A. Cells

containing both bait and prey plasmids were selected by growth on synthetic complete (SC) plates lacking tryptophan and leucine and spotted onto SC plates lacking tryptophan, leucine, and histidine; growth on the latter (scored after two days at 30˚C) indicates physical interaction between fusion proteins tested.

In Chapter 3, full-length or truncated versions of human TRIP or *Drosophila* NOPO were subcloned into pGBKT7 and subsequently transformed into yeast strain AH109 (Clontech). Full-length or truncated cDNAs of human *POLH*, *POLI*, *POLK*, and *REV1* or *Drosophila DNApol-eta*, *DNApol-iota*, and *dRev1* were subcloned into pGADT7 to encode a hybrid protein containing the Gal4 activation domain. Truncation constructs were made by PCR cloning. Prey constructs were transformed into strain Y187 and mated with yeast strain AH109 expressing a bait plasmid. Diploid cells expressing both bait and prey constructs were selected by growth on selective media lacking Leu and Trp (double dropout, DDO). Dilutions were made from a colony grown on DDO media resuspended in sterile water and serial diluted 1:10, 1:100 and 1:1000. Interactions were tested by spotting diluted cells onto TDO medium and scoring growth after 3 days at 30°C. Side by side plating on DDO was performed as a control.

DROSOPHILA DNA DAMAGE RESPONSE ASSAYS

Sensitivity of *nopo* larvae to hydroxyurea or irradiation was tested as described (Rickmyre et al., 2007).

BEHAVIORAL ASSAYS AND TDT MORPHOLOGY

To assess the visually mediated jump response, white-eyed control (w^{1118}) and mutant flies (two days old) were dark adapted, transferred without anesthesia to a Petri dish covered in vellum, and exposed to a "lights off" stimulus using an LED light apparatus as described (Fayyazuddin et al., 2006). Ten males per genotype were each tested in ten trials separated by 30 seconds. Climbing ability of adult males was assessed as described (Silva et al., 2004) with three replicates per genotype. *P*-values were obtained using two-tailed unpaired Student's *t*-tests. To visualize TDT muscle attachment sites, adult males (30 per genotype) were ventrally transilluminated with a dissecting microscope lamp as described (Edgecomb et al., 1993).

INNATE IMMUNITY ASSAY

Adult males (5-7 days old) were injected using a Drummond Nanoject with ~50 nanoliters of an overnight culture of *E. coli* resuspended in phosphate-buffered saline. Six hours later, RNA was isolated by homogenizing flies in STAT-60 buffer according to manufacturer's directions (Isotex Diagnostics). Following DNase treatment, cDNA was prepared by reverse transcription using Superscript II (Invitrogen). A *diptericin*-specific LUX primer (Invitrogen) was used to perform quantitative real-time PCR with the 7300 Real-Time PCR System (Applied Biosystems). *diptericin* levels were normalized to *Rp49* levels as an endogenous control. Results from three independent experiments were averaged and further normalized against buffer-injected Canton S flies. *P*-values were obtained using a two-tailed unpaired Student's *t*-test.

THE YEAST TWO-HYBRID SCREEN

Human *TRIP* cDNA was amplified by PCR and subcloned into pGBKT7 to encode a hybrid protein containing the DNA-binding domain of Gal4 (Clontech). Yeast cells of the AH109 strain were transformed with pGBKT7-hTRIP and mated with yeast cells pretransformed with a HeLa cDNA library according to manufacturer's directions (Clontech). Expression was determined by SDS-PAGE and immunoblotting, and X-α-galactosidase activity was assessed as a measure of transactivation. Transformants were selected on minimal media lacking His, Leu, and Trp (triple dropout, TDO) selection medium. Positive prey plasmids were sequenced and retested on TDO selection medium to confirm the interactions.

DROSOPHILA CELL CULTURE AND TRANSFECTIONS

Drosophila S2 cells were maintained in Schneider's media (Invitrogen) containing 10% fetal bovine serum. Plasmids generated by subcloning into tagged versions of pRmHa3 were transiently transfected into cells using the calcium phosphate method as previously described (March et al., 2010). ~24 hours after transfection, cells were washed and replated in fresh media, and expression was induced with 1 mM $CuSO_4$.

EXTRACT PREPARATION AND IMMUNOPRECIPITATION

For mammalian cell studies, cell lysates and immunoprecipitation were performed as previously described (Kim et al., 1999). HeLa cells were transiently transfected with 2 μg HA-POLH, HA-POLI, or FLAG-POLK and 1 μg MYC-hTRIP or empty vector. 24 hours after transfection, cells were washed with PBS and then lysed in non-denaturing

lysis buffer (NDLB; 1% Triton X-100, 150 mM NaCl, 10 mM TrisHCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, protease inhibitors). Lysates were centrifuged for 15 min at 13,000 rpm and were frozen at -80°C. Cell lysates containing 700 μg of total protein were incubated with shaking for 2 hours at 4°C with 50 μl of Protein A/G agarose beads (Santa Cruz). The beads were washed 3X with NDLB and 2X with PBS. Bound proteins were analyzed by SDS-PAGE and immunoblotting.

For *Drosophila* cell studies, cell lysates and immunoprecipitation were performed as above. S2 cells were transiently transfected with 3 μg pRmHa3-MYC-NOPO or empty vector and 5 μg pRmHa3-HA-DNApol-eta or pRMHa3-HA-DNApol-iota. 24 hours after induction with $CuSO_4$, cells were washed with PBS and then lysed in NDLB. Immunoprecipitation, SDS-PAGE, and immunoblotting were performed as described above.

For *Drosophila* embryo studies, embryos were dechorionated in 50% bleach and washed with distilled water. Embryos were lysed in NDLB by homogenization with a pestle. Lysates were centrifuged for 15 minutes at 13,000rpm and were frozen at -80 °C. For immunoprecipitation, embryo lysates containing 700 μg total protein were incubated with Protein A/G agarose beads (Santa Cruz) and anti-MYC antibodies (clone 9E10) and washed as described above. Bound proteins were analyzed by SDS-PAGE and immunoblotting.

UBIQUITYLATION ASSAYS

For mammalian cell studies, we used the previously established His_6-Ubiquitin method (Treier et al., 1994; Campanero and Flemington, 1997). ~$1X10^6$ HeLa cells were

transfected 16 hours after plating onto a 6 cm dish. Cells were transiently transfected with 1 μg Myc-hTRIP or empty vector and 2 μg HA-POLH, HA-POLK, or FLAG-POLI in the absence or presence of 1 μg pMT107 (gift of Dirk Bohmann and William Tansey), which encodes His_6-human ubiquitin. After 24 h of incubation with the DNA complexes, cells were harvested and histidine-tagged proteins purified on Nickel-nitrilotriacetic acid (Ni-NTA) agarose (Qiagen) exactly as previously described (Campanero and Flemington, 1997). Bound proteins were detected by SDS-PAGE and immunoblotting as described above.

For *Drosophila* S2 cell studies, we modified the method described above using a plasmid we generated to express Histidine-tagged ubiquitin in S2 cells, pRmHa3-His_6-ubiquitin. $3X10^6$ cells were transfected 16 hours after plating onto a 6 cm dish. Cells were transiently transfected with 3 μg pRmHa3-MYC-NOPO or empty vector and 5 μg pRmHa3-HA-DNApol-eta in the absence or presence of 2 μg pRmHa3-His_6-ubiquitin. 24 h after induction with $CuSO_4$, cells were harvested and histidine-tagged proteins purified on Ni-NTA agarose (Qiagen) as described above. Bound proteins were detected by SDS-PAGE and immunoblotting as described above.

For *Drosophila* embryo studies, we used transgenes expressing UASp-His_7-Ubiquitin (gift of Lynn Cooley) and UASp-HA-DNApol-eta. We expressed HA-DNApol-eta, in the presence or absence of His_7-ubiquitin, using the driver nanos-Gal4 in the female germ line of wild type or *nopo* mutant females. 0-3 hour embryos were collected, lysed under denaturing conditions as described above, and histidine-tagged proteins were purified on Ni-NTA agarose (Qiagen) as described above. Bound proteins were detected by SDS-PAGE and immunoblotting as described above.

UV TREATMENT OF CULTURED CELLS

HeLa cells were washed with PBS and treated with UV at 80-100% confluency. UV treatment was at 20 J/m^2. Recovery was in complete medium for 6 hours.

RNA EXTRACTION AND ANALYSIS

RNA was extracted from 0-3 hour embryos using RNA STAT-60 (Tel-Test, Inc.) and reverse transcribed (RT) using the High Capacity cDNA RT Kit (Applied Biosystems). Reactions lacking RT were performed as a control. Polymerase chain reaction (PCR) was performed using RT reaction products as template. PCR products were resolved by agarose gel electrophoresis.

GENERATION OF *DNApol-eta* NULL ALLELE

A *DNApol-eta*-null allele was generated by imprecise excision of *P*-element *EY07711*. The 2.1 kb deletion *DNApol-eta*^{Exc176} lacks part of the 5'-UTR and exons encoding residues 1-598.

CHAPTER VI

CONCLUDING REMARKS

SUMMARY

In a screen for cell-cycle regulators, we identified a *Drosophila* maternal effect-lethal mutant that we named "*no poles*" (*nopo*). Embryos from *nopo* females undergo mitotic arrest with barrel-shaped, acentrosomal spindles during the rapid S-M cycles of syncytial embryogenesis. We identified *CG5140*, which encodes a candidate RING domain-containing E3 ubiquitin ligase, as the *nopo* gene. A conserved residue in the RING domain is altered in our EMS-mutagenized allele of *nopo*, suggesting that E3 ligase activity is critical for NOPO function. We showed that mutation of a DNA checkpoint kinase, Chk2, suppresses the spindle and developmental defects of *nopo*-derived embryos, revealing that activation of a DNA checkpoint operational in early embryos contributes significantly to the *nopo* phenotype. Chk2-mediated mitotic arrest has been previously shown to occur in response to mitotic entry with DNA damage or incompletely replicated DNA. Syncytial embryos lacking NOPO exhibit a shorter interphase during cycle 11, suggesting that they may enter mitosis prior to completion of DNA replication. We showed that Bendless (BEN), an E2 ubiquitin conjugating enzyme, interacts with NOPO in a yeast two-hybrid assay; furthermore, *ben*-derived embryos arrest with a *nopo*-like phenotype during syncytial divisions. These data support our model that an E2-E3 ubiquitination complex consisting of BEN-UEV1A (E2

heterodimer) and NOPO (E3 ligase) is required for preservation of genomic integrity during early embryogenesis.

NOPO is the *Drosophila* homolog of mammalian TRAF-interacting protein (TRIP). NOPO and TRIP contain highly similar RING domains that closely resemble that of known E3 ubiquitin ligases. We sought to elucidate the mechanism by which NOPO/TRIP promotes genomic stability by performing a yeast two-hybrid screen to identify NOPO/TRIP interactors and/or substrates. We identified several interesting proteins that are involved in immune signaling, apoptosis and the cell cycle. One protein is a member of a family of non-canonical DNA polymerases that facilitate the replicative bypass of damaged DNA (translesion synthesis, TLS). In mammals, there are four Y-family DNA polymerases: POLH, POLI, POLK and REV1. Mutation of one of the human Y-family polymerases, POLH, results in a variant form of Xeroderma Pigmentosum, a disease characterized by UV sensitivity and skin cancer. We showed that TRIP interacts with all of these TLS polymerases via yeast two-hybrid assays and co-immunoprecipitation from cultured mammalian cells.

The Y-family polymerases conserved in *Drosophila* are *DNApol-eta*, *DNApol-iota* and *Rev1*. We showed that *Drosophila* NOPO interacts with these Y polymerases via yeast two-hybrid assays as well as co-immunoprecipitation from cultured *Drosophila* S2 cells and early embryos. We also showed that TRIP and NOPO E3 ligases enhance the ubiquitylation of the Y-family polymerases in cultured mammalian and *Drosophila* cells, respectively. Furthermore, co-expression of TRIP and POLH in cultured mammalian cells alters POLH's localization by recruiting it to the nucleolus with TRIP.

In *C. elegans*, POLH-1, the DNApol-eta homolog, has been proposed to prevent stalling of replication forks during the early embryonic cell cycles by quickly responding to and bypassing lesions, thereby keeping these rapid cell cycles on schedule. We generated a null *DNApol-eta* mutant line and found that these mutant females have reduced fertility and produce embryos with *nopo*-like spindle defects. Together, these data suggest that DNApol-eta has a unique role during early *Drosophila* embryogenesis to promote cell-cycle progression and that NOPO regulates its activity. We hypothesize that NOPO ubiquitylates Y-family DNA polymerases during S-phase of early *Drosophila* embryogenesis so as to control their localization and interaction with chromatin at sites of DNA damage.

DISCUSSION AND FUTURE DIRECTIONS

TRIP is a RING-dependent E3 ubiquitin ligase, but ubiquitylation substrates of TRIP have not yet been reported (Besse et al., 2007). TRIP is also implicated in apoptosis; a recent study showed that the tumor suppressor Syk phosphorylates TRIP and that overexpression of TRIP sensitizes cells to TNF-induced apoptosis (Zhou and Geahlen, 2009). TRIP plays a TNF-independent role in cell proliferation: when TRIP is knocked down in keratinocytes, cells exhibit an NF-κB-independent G1/S phase cell-cycle arrest (Almeida et al., 2011).

In future experiments, it would be interesting to use cultured keratinocytes to study the role of endogenous TRIP in mammalian cells. Because we have not been able to detect *TRIP* expression in the cultured mammalian cells that we had readily available in the lab (data not shown), we cannot knock down *TRIP* expression by RNAi in our cell

lines to study its loss of function phenotype. Performing *TRIP* RNAi experiments in a cell line expressing endogenous *TRIP* would be extremely useful to study the ubiquitylation and regulation of the Y-family DNA polymerases. These experiments could address the specificity of TRIP's ubiquitylation of the Y-family polymerases and may help to identify other potential substrates of TRIP *in vivo*.

Direct confirmation for ubiquitylation of the Y-family DNA polymerases by NOPO/TRIP would require *in vitro* approaches. This would involve adding *in vitro*-translated, radiolabeled substrate proteins to purified E1, the E2 (BEN-UEV1A or UBC13-UEV1A), NOPO or TRIP, Ub, and ATP. Following incubation, the readout for ubiquitylation of the Y polymerase substrates would be to see an upward mobility shift/laddering of the radiolabeled proteins, consistent with ubiquitylation. This approach could also establish whether BEN-UEV1A (or UBC13-UEV1A) is the E2 heterodimer involved in the ubiquitylation of the Y polymerases by NOPO/TRIP.

Once *in vitro* assays are established, there are many other important biochemical questions that could be addressed. First, we could attempt to identify the lysine residue(s) that is/are ubiquitylated on the Y polymerases by NOPO/TRIP. We could also determine the type of polyubiquitin linkage on these polymerases. This can be done using established ubiquitin mutants (i.e. K0, K11R, K48R, K63R) and observing the ubiquitylation pattern of the substrate in the presence of NOPO/TRIP compared to wild-type ubiquitin. These experiments are technically difficult to perform in cultured cells, likely due to high levels of endogenously expressed ubiquitin, which may compete with the transfected mutant forms for incorporation into substrates. Such restrictions in cultured cells make the suggested *in vitro* approaches invaluable.

We predict that NOPO/TRIP E3s mediate assembly of K63-linked ubiquitin chains on their substrates to preserve genomic integrity. While K48-linked ubiquitin chains have established roles in targeting proteins for proteasome-mediated degradation, K63-linked ubiquitin chains are not thought to act as proteolytic signals (Pickart and Fushman, 2004). In the presence of NOPO/TRIP, we do not see a detectable decrease in the levels of Y polymerases. Furthermore, in budding yeast and mammalian cells, the E2 heterodimers Ubc13-Mms2 and Ubc13-Uev1A mediate K63 ubiquitin chain assembly on target proteins. Additional investigation into the type of polyubiquitin linkage will be critical in elucidating the mechanism by which NOPO/TRIP E3s regulate cell-cycle progression.

Another way to establish that NOPO/TRIP is responsible for ubiquitylation of the Y-family polymerases is to specifically perturb the E3 ligase activity of TRIP by making mutations in the E3. The RING domain of E3 ubiquitin ligases is likely involved in mediating protein-protein interactions, most commonly to its partner E2(s). Therefore, by mutating the RING domain, the E3 will no longer be able to bind the E2(s), and ubiquitin cannot be transferred to the substrate protein. We have generated several constructs to address this issue: (1) a D12K mutation in TRIP alters the evolutionarily conserved residue that is mutated in our *nopo*^{Z1447} mutants (E11K), (2) a C7A mutation in the first cysteine of TRIP's RING domain, previously reported to disrupt mouse TRIP's E3 ligase function *in vitro*, (3) a I9A mutation that is predicted to disrupt the conformation of the RING domain, and (4) a delta RING mutant in which the entire RING domain is deleted. The individual point mutations (1-3), however, did not change TRIP's E3 ligase activity in our cultured cell assays, while the delta RING mutant TRIP (4) was unstable when

overexpressed in human cells (data not shown). In order to completely disrupt TRIP's E3 activity, we predict that we must mutate all of the cysteine and histidine residues within TRIP's RING domain. Once these mutations are made, TRIP's E3 ligase function can be further tested. Such experiments would also be interesting to perform for NOPO in cultured *Drosophila* S2 cells. We have shown that the E11K mutation of NOPO disrupts its interaction with BEN via yeast two-hybrid assays, so we would predict that this mutation would also disrupt its E3 ligase activity when overexpressed in S2 cells (Merkle et al., 2009). The results of these experiments would be very informative to further understand NOPO/TRIP's E3 ligase activity.

There is evidence that mammalian TRIP is important during development. Mouse TRIP is needed for early embryonic development: homozygous mutant mouse embryos die shortly after implantation due to proliferation defects and excessive cell death (Park et al., 2007). This early embryonic arrest occurs prior to when TNF signaling is first required during embryogenesis, suggesting that TRIP has roles outside of its proposed TNF-signaling functions (Gerhart, 1999). Another developmental role suggested for TRIP involves TNF/NF-κB-dependent sexual dimorphism in developing neurons (Krishnan et al., 2009). This study showed an upregulation of TRIP in the developing male anteroventral periventricular (AVPV) nucleus, a region that is larger in females and critical for regulating the release of female-specific luteinizing hormone (LH). The size difference in male versus female AVPV nuclei results from apoptosis in developing male neurons. Although some of the roles for TRIP may occur through TNF signaling, others clearly are not. Further investigation is likely to reveal new roles for TRIP in TNF-dependent and –independent developmental processes.

We have begun preliminary studies to look at the role of TRIP during vertebrate development using early *Xenopus* embryos. Antisense morpholino-mediated depletion of TRIP in *Xenopus* embryos results in an embryonic developmental arrest (our unpublished data), confirming that TRIP is needed during early embryonic development in vertebrates. Future efforts will focus on the use of developmental markers to further elucidate TRIP's role in *Xenopus* embryogenesis.

As discussed earlier, it will be important to study the loss of function phenotype of TRIP in order to better understand its roles. Other than RNAi, another way to address this would be to obtain the TRIP mutant mice generated by Park et al. (2007) and attempt to isolate MEFs (mouse embryonic fibroblasts) from the homozygous mutant embryos. These *TRIP*-null MEFs would be an ideal system to look at the regulation and localization of the Y polymerases in the absence of TRIP compared to wild-type mouse MEFs. A major caveat of this approach, however, is that MEFs are difficult to transiently transfect, and endogenous Y polymerases have been difficult to detect by immunostaining. If *TRIP*-null MEFs could be isolated and cultured, it would be very interesting to investigate TRIP's regulation and ubiquitylation of the Y polymerases in mammalian embryonic cells. We could then determine if TRIP's role in mammalian embryogenesis is analogous to that of NOPO's role during *Drosophila* embryogenesis.

Future efforts should also focus on dissecting the interactions of TRIP with the candidate hits identified in our yeast two-hybrid screen. One group of interactors is associated with apoptosis and NF-κB signaling. There is also evidence that TRIP has roles outside of TNF-signaling. We identified MDM2 and LZAP in our yeast two-hybrid screen for TRIP interactors, and we confirmed these interactions by demonstrating their

coimmunoprecipitation from cultured mammalian cells. The developmental requirement for mammalian TRIP and *Drosophila* NOPO may be linked to MDM2 and/or LZAP because MDM2 and LZAP mutant mice also die in early embryonic development. It will be interesting to see if any of the proposed roles for TRIP in apoptosis and cell proliferation involve its interaction with LZAP and/or MDM2.

It will also be important to determine if TRIP's interactions with LZAP and MDM2 are that of an E3 ligase and its substrates. Since all of these proteins have been implicated in cell-cycle progression, genome maintenance and development, uncovering the mechanisms by which these proteins regulate these processes will be extremely informative. Although there is no obvious MDM2 homolog in *Drosophila*, by sequence similarity alone, p53 levels are regulated in flies, suggesting that an MDM2 may be functionally conserved in *Drosophila* (Sekelsky et al., 2000; Jin et al., 2000; Brodsky et al., 2004). We are particularly interested in future efforts to identify a *Drosophila* MDM2 homolog and to study the potential roles of NOPO in regulating MDM2 and LZAP during early *Drosophila* embryogenesis.

Although the function of TRIP at the nucleolus has yet to be identified, this nuclear compartment has been shown to be a site at which proteins are sequestered and held inactive (Boisvert et al., 2007). The nucleolus is the site of ribosome biosynthesis and plays roles in other cellular processes. In the presence of DNA damage, MDM2 is sequestered to the nucleolus, so it cannot ubiquitylate and degrade the tumor suppressor p53 (Bond et al., 2005). Based on TRIP's localization and interaction with MDM2, we favor a model in which TRIP ubiquitylates and sequesters its substrates to the nucleolus under certain cellular or environmental conditions.

We have yet to fully elucidate the role of NOPO in regulating cell-cycle progression and genome maintenance during early *Drosophila* embryogenesis. We predict that NOPO's function is conserved based on rescue of the sterility of *nopo* mutant females with a transgene expressing human *TRIP*. We predict that NOPO's ubiquitylation of its substrates results in K63-liked polyubiquitylation and a subsequent change in its substrate's subcellular localization. We base our model on results obtained from *C. elegans* in which DNApol-eta also has a unique role during early embryogenesis to control cell-cycle timing. We predict that *Drosophila* DNApol-eta also has a unique role in early embryogenesis and that its activity and association with chromatin is regulated by NOPO. We hypothesize that NOPO ubiquitylates Y-family DNA polymerases during S-phase of early *Drosophila* embryogenesis and that ubiquitylation of these polymerases by NOPO regulates their localization and interaction with chromatin. More specifically, we predict that NOPO-dependent ubiquitylation of the Y-family polymerases results in their sequestration away from the chromatin so that canonical polymerases can bind to the chromatin and promote DNA synthesis.

SIGNIFICANCE

Cell division is essential for the development of all tissues and organs within multicellular organisms. It is a fundamental facet of life, and the erroneous segregation of genetic information may result in developmental disorders or disease states, such as cancer. If the regulation of cell-cycle components is perturbed during critical developmental stages, sterility or developmental disorders may result. Cancer can result from the mutation or dysfunction of key cell-cycle regulators. Spontaneous mutations

arise with each cell division, and if those mutations are not corrected or if the mutated cells are not prevented from dividing, this may result in unregulated growth. A more complete understanding of the cell-cycle machinery and how developmental defects and cancers progress will lead to more effective therapeutics in order to fight these diseases.

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