DNase II mediates a parthanatos-like developmental cell death pathway in Drosophila primordial germ cells

Lama Tarayrah-Ibraheim, Elital Chass Maurice, Guy Hadary, Sharon Ben-Hur, Alina Kolpakova, Tslil Braun, Yoav Peleg, Keren Yacobi-Sharon & Eli Arama

During Drosophila embryonic development, cell death eliminates 30% of the primordial germ cells (PGCs). Inhibiting apoptosis does not prevent PGC death, suggesting a divergence from the conventional apoptotic program. Here, we demonstrate that PGCs normally activate an intrinsic alternative cell death (ACD) pathway mediated by DNase II release from lysosomes, leading to nuclear translocation and subsequent DNA double-strand breaks (DSBs). DSBs activate the DNA damage-sensing enzyme, Poly(ADP-ribose) (PAR) polymerase-1 (PARP-1) and the ATR/Chk1 branch of the DNA damage response. PARP-1 and DNase II engage in a positive feedback amplification loop mediated by the release of PAR polymers from the nucleus and the nuclear accumulation of DNase II in an AIF- and CypA-dependent manner, ultimately resulting in PGC death. Given the anatomical and molecular similarities with an ACD pathway called parthanatos, these findings reveal a parthanatos-like cell death pathway active during Drosophila development.
Programmed cell death (PCD) is a cell suicide process fundamental for the development and homeostasis of the organism, and malfunction of this process is associated with the pathogenesis of multiple diseases5–8. Apoptosis, the major form of PCD during animal development, is mediated by the activation of a unique family of cysteine proteases called caspases5–8. However, studies in the past several decades suggest that cells can sometimes activate caspase-independent alternative cell death (ACD) pathways9, 10. About a dozen mechanistically distinct ACD pathways have been described in different experimental systems and studied mainly under non-physiological conditions11. ACDs have received considerable attention in recent years because of their involvement in the pathogenesis of a variety of diseases, leading to the perception that intervention in these pathways could be a promising avenue for developing new therapeutic strategies12–14. However, many questions regarding the in vivo biology of ACD pathways remain open, as it is inevitable that studies in cell culture cannot fully reproduce the complexity of the in vivo reality, leading, at times, to inaccurate notions15. On the other hand, although fascinating, only a few physiological ACD paradigms have been described in complex metazoan organisms, e.g. linker cell death in C. elegans16, 17, autophagic cell death in the Drosophila salivary glands18 and mid gut19, germ cell death of Drosophila spermagonia20, 21, and phagoptosis of the germline nurse cells in Drosophila22. Furthermore, the connections, if any, between these paradigms and the non-physiological ACDs are not always clear, and for most of the ACDs, an equivalent physiological system has not been identified11–13.

Parthanatos is an ACD pathway that has been almost exclusively investigated under non-physiological conditions in mammalian cells, and is distinct from apoptosis, autophagy or necrosis at both the molecular and biochemical level23, 24. Parthanatos is triggered by overexpression (OE) of the DNA damage-sensing enzyme, Poly(ADP-ribose) polymerase-1 (PARP-1), or its activation following DNA damage caused by genotoxic stress or excitotoxicity25–27. Following PARP-1 activation, the mitochondrial protein, Apoptosis Inducing Factor (AIF), is released to the cytosol, where it associates with and facilitates nuclear translocation of the Deoxyribonuclease (DNase) Macrophage Migration Inhibitory Factor (MIF)26–28. Parthanatos has been implicated in the pathogenesis of several important diseases, including Parkinson’s disease, stroke, heart attack, diabetes, and ischemia reperfusion injury in numerous tissues29–34. However, it remains unknown whether cell death by parthanatos is only limited to stress or pathological conditions, or if it might also operate during normal development and homeostasis of the organism.

Here, we report the characterization of a caspase-independent form of PCD in Drosophila, through which about 30% of the primordial germ cells (PGCs) are normally eliminated during early embryogenesis. Detailed analysis of the underlying mechanisms revealed striking resemblance to parthanatos; PGC death pathway is mediated by AIF-dependent nuclear translocation of the lysosomal nuclease, DNase II, and the consequent DNA damage induced PARP-1 activation. Given the rarity of developmental ACD pathways identified in metazoan model organisms, these findings may extend our understanding of the connections between the non-physiological and physiological ACD pathways, as well as when and why ACD may sometimes be advantageous over apoptosis.

Results

PGCs undergo caspase-independent cell death. Primordial germ cells (PGCs) arise from germline progenitors, called pole cells, initially found at the posterior pole of the early Drosophila embryo32. During gastrulation, the PGCs are carried as a loose cluster into the posterior midgut pocket (embryonic stage [ES] 9) from where they migrate across the midgut epithelium near the embryo midline (ES 10; Fig. 1a). The PGCs disperse from this cluster, sort bilaterally (ES 11) and migrate toward somatic gonadal precursors (ES 12), eventually compacting into two round gonads (ES 13; Fig. 1a)33, 34. However, not all PGCs specified at early embryogenesis successfully migrate from the midline position to the gonads, and several reports showed that these cells are eliminated by cell death34–39. Interestingly, previous attempts to block PGC death through inhibition of apoptosis, including genetic inactivation of the Inhibitor of apoptosis (IAP) protein antagonists (the reaper family genes) and overexpression (OE) of the baculovirus effector caspase inhibitor protein p35, and the Drosophila IAP proteins, Diap1 and Diap2, as well as OE of a dominant-negative (DN) form of the Drosophila caspase-9 homolog Dronc, have all failed in this regard, suggesting divergence from the conventional apoptotic program35–37, 39.

To explore the mechanisms underlying PGC death, we implemented a unified quantitative method which has been used by several groups to evaluate the levels of PGC death in different Drosophila strains and mutants35–37, 39. The basis for this approach is that the number of PGCs is relatively small and can be readily visualized by staining with an anti-Vasa antibody, allowing for manual counting of their numbers before cell death induction, and when all the PGCs are still dispersed at the embryo midline (ES 10), and after cell death of the aberrantly migrating PGCs is almost completed (ES 13; Fig. 1a, b). Since during these stages, the PGCs neither divide40 nor transdifferentiate41, the difference in the number of PGCs between ES 10 and 13 accurately reflects the number of dying PGCs. To define the average levels of PGC death, we examined embryos from three different standard D. melanogaster laboratory reference strains, yellow1 white1118 (yw), Oregon R (OR), and Canton-S (CS). Whereas within each of these fly strains, the number of PGCs at ES 10 was relatively constant (n = 3), it could vary considerably among embryos of the different strains at the same ES (Fig. 1c). In contrast, the relative decrease in the number of PGCs between ES 10 and 13 (~30%) was highly conserved among all of the examined fly strains, allowing for comparative measurements of PGC death levels among different genetic backgrounds (Fig. 1c). The dying midline PGCs can sometimes be also directly visualized at advanced demolition stages by virtue of their condensed and distorted morphology, as well as the reduction in the Vasa staining signal (arrows in Fig. 1b). Since by ES 13 only a few such dying PGCs could be detected, implying that most of the midline PGCs already undergo cell death at earlier stages, we examined the time window during which most of the midline PGCs undergo cell death by counting the PGC numbers at ES 10, 11, 12, and 13. Whereas condensed and distorted dying PGCs could be detected at all these stages, the majority underwent cell death between ES 10 and 12 with a small peak at ES 11 (Fig. 1d). These findings are also in agreement with a previous report showing that most of PGC death occurs between ES 10 and 1139.

Collectively, we conclude that between ES 10 and 13, the PGCs are divided into two subsets, one of which migrates to the gonads (encompassing around two-thirds of the PGCs and are henceforth referred to as the gonadal subset), and the second of which remains near the midline and undergoes cell death in an asynchronous manner (encompassing about one-third of the total PGCs and are henceforth referred to as the midline subset).

Using this assay, we initially wanted to confirm that PGC death can indeed proceed when caspase activity is compromised. For this, we used the nos-Gal4-VP16 driver42 to overexpress potent inhibitors of the apoptotic caspase activity
and/or specific RNA interference transgenes (Ri), as well as used genetic mutants when possible, in order to inactivate the seven apoptotic and non-apoptotic Drosophila caspases. As previously reported, OE of the potent caspase inhibitory proteins, p35, Dia1 and DroncDN, all failed to attenuate PGC death (Fig. 1e, f, i). Furthermore, PGC death proceeded normally when each of the seven Drosophila caspases, as well as the major apoptotic corpses engulfment receptor homolog of CED-1, Draper (drprΔ5)43, were inactivated, either following PGC-specific knockdown or in maternally and/or zygotically mutant embryos (Fig. 1g–j and Supplementary Fig. 1m). Altogether, these findings confirm that PGC death is a bona fide caspase-independent cell death pathway.

DNase II mediates PGC death but not PGC migration. To identify the pathway by which PGCs undergo cell death, we first tested for possible involvement of an ACD pathway called germ cell death (GCD), which operates in the adult Drosophila testis20. We inactivated four genes encoding major mediators of GCD: The mitochondrial serine protease HtrA2/Omi and Endonuclease G (EndoG), and the lysosomal endonuclease DNase II and protease Cathepsin D (CathD). Critically, whereas both maternal and zygotic inactivation of Omi, EndoG, and CathD had no significant effects on the levels of PGC death (Supplementary Fig. 1a–d, g), a complete block of PGC death was detected in embryos maternally, but not zygotically, mutant for a strong hypomorphic allele of DNase II (dnaseIIlo), as well as upon
PGC-specific knockdown of dnasII (dnasII\(^{20i}\); Fig. 2a–d). Importantly, inactivation of DNase II had no effect on normal migration and incorporation to the gonads of the gonadal subset (Fig. 2a, c, e). Furthermore, during subsequent embryonic stages, the ectopically surviving midline subset were scattered randomly in the posterior half of the mutant embryos, and could be readily detected even at ES 17 when the larval structures are already formed (Fig. 2f–j).

Taken together, these observations suggest that maternal dnasII is cell autonomously required for PGC death, and that in its absence, the midline subset of PGCs survive throughout embryogenesis.

Previous studies have coupled between PGC migration across the midgut at ES 9/10 and the competence to undergo cell death. In particular, it was shown that in embryos mutant for regulators of PGC migration, the PGCs remained inside the gut and were resistant to cell death\(^{34}\). To explore for possible effects of DNase II inactivation on the ability of the midline subset of PGCs to transverse the midgut, we co-stained dnasII mutant embryos with anti-Hb9 antibodies, which label the posterior midgut primordium\(^{45}\), and with anti-Vasa antibodies to reveal the PGCs. Whereas PGCs that cross the midgut epithelium could be detected already at ES 10, it was still difficult to differentiate between the different subsets of PGCs at that stage (Supplementary Fig. 2a). However, starting at ES 11 and onward, the gonadal subset of PGCs, which already started sorting into two groups, and the surviving midline subset, became clearly distinct, and both were positioned outside of the midgut (Supplementary Fig. 2b–d). Therefore, migration of the midline PGC subset across the midgut is unaffected in the dnasII mutants.

DNase II is involved in PGC death upon Wunens inactivation.

Once PGCs transverse the midgut, they become dependent on survival signals provided maternally by the lipid phosphate phosphatases (LPPhs), Wunen (Wun), and Wun, which act redundantly in the germ cells and the soma to regulate PGC migration and cell death\(^{35,36,44}\). Interestingly, it has been shown that in embryos maternally mutant for wun\(^{2}\) alone or double mutant for wun\(^{2}\) and \(wingless\), PGCs undergo precocious non-apoptotic cell death, eliminating about 50% of the PGCs in the wun\(^{2}\) mutants and almost all the PGCs in the double mutants\(^{35,36,44}\). We, therefore, asked whether DNase II might also mediate PGC death induced by the lack of maternal wunens. Analyzing a mutant allele of wun, which was suggested to also act as a dominant-negative allele of \(wun\), dubbed wun\(^{N14}\) (henceforth referred to as wun\(^{−/−}\))\(^{35,44}\), revealed that 40% of the maternally mutant wun\(^{−/−}\) embryos displayed no PGCs already at ES 9/10, suggesting that they might have died or transdifferentiated early in development (Fig. 3a, b, g). We then compared PGC numbers in ES 13 and 14 embryos laid by wun\(^{−}\)/−\) deficient mothers and by wun\(^{−}\)/−\) and dnasII\(^{−}\) double mutant mothers. Significantly, whereas half of the wun\(^{−}\)/−\) embryos contained 2 PGCs on average, and the other half had zero PGCs, 70% of the double mutant embryos contained 6 PGCs on average while 30% had zero PGCs (Fig. 3c–g). Since already at ES 9/10, 40% of the wun\(^{−}\)/−\) embryos had no PGCs, these findings suggest that essentially all the double mutant embryos which started with regular numbers of PGCs at ES 9/10 contained surviving PGCs. Furthermore, the Vasa staining signal was much more intense in the double mutant surviving PGCs as compared with the wun\(^{−}\)/−\) PGCs, further implying that the latter might be dying or transdifferentiating (Fig. 3c–f). Of interest, almost all of the surviving PGCs in the double mutants migrated to the gonads, further demonstrating that DNase II is not involved in PGC migration (Fig. 3d, f). Moreover, in a few double mutant embryos, the average number of surviving PGCs which migrated to the gonads (34 PGCs on average) was almost identical to the number of PGCs at ES 9/10 (33 PGCs on average), suggesting that in the absence of the wunens, both the gonadal and midline subsets of surviving PGCs have similar capacity to migrate to the gonads (Fig. 3d, f, g). Finally, not all PGCs survived in the double mutants, which may be attributed to a potent cell death signal triggered in the absence of survival signal from Wunens, and to the fact that the dnasII\(^{−}\) mutant is a hypomorph.

Overall, these observations suggest that DNase II is involved in PGC death induced by wunens deficiency, revealing a direct link between the developmental cell death pathway and the survival signaling pathway of PGC death.

PGC death requires lysosomal leakage and is independent of macroautophagy.

To further explore the role of the lysosomal pathway in PGC death, we inactivated several lysosomal biogenesis proteins and catalytic proteases, and examined their effects on PGC death. PGC-specific knockdowns of the lysosomal cysteine proteases CathB and CathL, both significantly decreased PGC death levels (Supplementary Fig. 1d–g). Furthermore, PGC-specific knockdowns of the genetically linked lysosomal biogenesis proteins, the Vps18p homolog Deep-orange (Dor) and the Vps33A homolog Carnation (Car), completely blocked PGC death (Fig. 2k and Supplementary Fig. 1i). These observations imply that functional lysosomes mediate PGC death.

Two major mechanisms for the involvement of lysosomes in ACD-associated cellular degradation have been proposed: Sequestration of cellular contents to lysosomes by autophagy, or
spillage of lysosomal catabolic enzymes to the cytosol, following
the induction of lysosomal membrane permeabilization
(LMP). We first tested for possible involvement of the
autophagic pathway in PGC death. However, no significant
effect on PGC death levels was detected when autophagy was
genetically compromised, including in maternally mutant embryos trans-heterozygous for two null atg7 alleles (atg7d77
and atg7d14), upon PGC-specific ectopic expression of a
dominant-negative form of Atg1 (atg1KQ13A), or following
RNAi-mediated knockdowns of atg7, atg5, atg6 and atg8a/LC3
(Supplementary Fig. 1j–l). In contrast, genetic inhibition of
LMP, achieved by interference with several pathways previously
reported to be involved in this process (Supplementary Fig. 3a;
also summarized in ref.47), resulted in pronounced attenuation
of PGC death (Supplementary Fig. 3b–f). Taken together, we
conclude that the lysosomes are major mediators of PGC death,
variably functioning through the release of their contents.

Drosophila p53 mutants were previously shown to be defective
in developmental PGC death, an effect that was attributed to both
maternal and zygotic expressions of p53, but the underlying
mechanisms remained elusive.39 In light of previous reports
implicating p53 as a positive regulator of LMP in mammalian
cells47, we monitored PGC death in ES 10 and 13 embryos both
maternal and zygotic homozygous mutants for the
p53 null allele, p535A-1-4, and in p53 knockdown embryos. Whereas similar to
the previous report, inactivation of p53 in the mutant and the
knockdown embryos attenuated PGC death39, this effect was only
detected in 57% of the embryos (Fig. 3h–j). Interestingly, some of
the p53 mutant embryos displayed more PGCs at ES 13 (a maximum of 36 PGCs) as compared with the same mutants at ES 10 (a maximum of 29 PGCs), implying that some of the PGCs may undergo cell division between ES 10 and 13 in these mutants. We therefore conclude that p53 may be involved in several aspects of PGC biology, including PGC death.

**DNase II nuclear translocation requires functional genetic LMP pathways.** Our observations suggest a cell autonomous function for DNase II during PGC death, which implies translocation of this lysosomal nuclease to the nucleus. To test this hypothesis, we generated specific polyclonal antibodies against full-length *Drosophila* DNase II. The capacity of these antibodies to detect DNase II in situ was confirmed by staining WT and *dnaseII* mutant embryos, showing that the specific DNase II signal in WT PGCs was abolished in the mutant (Fig. 4a, b). In WT embryos, DNase II was highly expressed in all the PGCs at ES 10, such that these cells could be clearly distinguished from the other cells of the embryo by virtue of the strong DNase II signal (Fig. 4a). Closer examination of stained ES 10 PGCs revealed that in the majority of the PGCs, DNase II was confined to small cytoplasmic vacuoles (Supplementary Fig. 4a, b), which we confirmed to be the lysosomes by co-staining for the lysosomal small GTPase Arl8 (Fig. 4c). Importantly, whereas DNase II that is exiting the lysosomes could be already detected in some of the midline PGCs with regular morphology (revealed by partial mislocalization with Arl8; Fig. 4d), dying PGCs, revealed by their condensed and distorted morphology, displayed strong and almost complete nuclear DNase II localization, implying that translocation of DNase II to the nucleus may constitute one of the earliest events of PGC death (Fig. 4e, f, Supplementary Movie 1, and Supplementary Fig. 4a, c).

In order to translocate to the nucleus, DNase II must be released from the lysosomes. We therefore examined whether DNase II translocation to the nucleus might be affected in the LMP deficient mutants. Staining these mutants, as well as the p53 mutant and knockdown embryos for DNase II revealed that all the ectopically surviving midline PGCs in these mutants displayed non-nuclear DNase II at ES 13 (Fig. 3k, l and Supplementary Fig. 3g–i). In contrast, 70% of the dying midline PGCs in WT embryos at ES 11 displayed nuclear DNase II (Supplementary Fig. 3i). Note that we compared between embryos of different developmental stages (WT, ES 11; mutants ES 13), as most of the dying PGCs in WT embryos are detected at ES 11 (Fig. 1d), while in the mutants, the ectopically surviving midline PGCs accumulate by ES 13. Overall, these findings demonstrate that DNase II nuclear translocation is dependent upon functional genetic LMP pathways.

**PGC death is associated with DNase II-dependent DNA breaks.** Optimal function of the lysosomal hydrolyses usually requires the low pH environment of the lysosomes. We therefore asked whether DNase II might still be functional in the nuclei of dying PGCs. For this, we examined whether PGC death is associated with DNA fragmentation, and if so, whether this might be dependent on DNase II. Examining nuclei of dying PGCs stained with the DNA fluorescent dye Hoechst, revealed gradual loss of the fluorescent signal, concomitant with the progression in cell death and the prominent of nuclear DNase II, suggesting that the DNA in the dying PGCs is highly fragmented (Fig. 5a and Supplementary Movie 1). Furthermore, at around early-to-mid cell demolition stages, the dying PGCs stained positively with specific antibodies against phosphorylated histone H2A variant (γ-H2Av; the *Drosophila* equivalent of the mammalian γ-H2AX), an early response modification to the presence of DNA double-strand breaks (DSBs) (Fig. 5b, d),[49] Importantly, no such staining was detected in ectopically surviving PGCs at the midline of *dnaseII* mutant embryos, implying that self-inflicted DNA double-strand breaks are mediated by DNase II during PGC death (Fig. 5c, d). It is interesting to note that although detected at a very low frequency, a few gonadal PGCs that are positive to γ-H2Av are also observed, which is consistent with a report observing 1–2 PGCs (on average) undergoing cell death in the gonads.[37]

A classical assay for direct in situ detection of DNA fragmentation during apoptosis is the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL). However, consistent with previous reports indicating that cells dying in response to Wunens-mediated signals were negative for TUNEL staining,[35,37], the developmentally dying PGCs were completely negative for TUNEL during the entire death process, although they displayed pronounced TUNEL labeling when induced to undergo precocious apoptosis by OE of the proapoptotic reaper family gene hid (Supplementary Fig. 5a, b). These observations suggest that PGC death is normally associated with DNA cuts distinct from those generated during apoptosis.
Whereas the TUNEL assay is designed to label DNA fragments with exposed 3’ hydroxyl (OH) groups generated by type I DNases, labeling of DNA fragments with exposed 5’-OH and 3’-PO4, which are produced by type II DNases (such as DNase II and Topoisomerase I [Top I]), requires a distinct assay based on Top I-mediated ligation of a fluorescently labeled oligonucleotide. Indeed, applying this assay to WT embryos revealed specific nuclear labeling in some of the dying midline PGCs, and not in the gonadal PGCs (Fig. 5e). Moreover, in the dnaseII mutant embryos, no labeling was detected in the ectopically surviving midline PGCs, consistent with direct involvement of DNase II in eliciting this DNA fragmentation (Fig. 5f). Consistently, no Top I-associated labeling was detected in PGCs that were induced to undergo precocious apoptosis, confirming the specificity of this assay for DNase II type cuts (Fig. 5g).

DNA damage and activation of the DDR pathway mediate PGC death. As opposed to apoptosis, in which DNA fragmentation marks the end-stage and ultimate demise of the cell, our findings suggest that the DNase II mediated self-inflicted DNA double-strand breaks is an early step in triggering PGC death. To independently test this idea, we sought to induce mild DNA damage in the PGCs by taking advantage of the Chlamydomonas reinhardtii homing endonuclease I-CreI, which was shown to induce multiple DNA breaks at a defined location on the Drosophila sex
Fig. 3 The interplay between DNase II and Wunens in PGC survival, and DNase II and p53 in PGC death. a–g The dnaseII<sup>lo</sup> mutant partially attenuates PGC death induced by the lack of maternal wunens. Shown are representative images of embryos maternally lacking the wunens (laid by wun<sup>2M</sup>/Df mutant mothers) at ES 10 (a, b), ES 13 (c) and ES 14 (e), as well as embryos maternally double mutant for both wunens and dnaseII at ES 13 (d) and ES 14 (f), stained to visualize the PGCs (Vasa; red). Asterisks indicate gonadal PGCs. Scale bars, 50 µm. The corresponding quantifications of total PGC numbers as compared to WT embryos are shown in (g). Mean PGC number is shown as a line in the middle of each data set, each dot is a single embryo to reflect n number, where n = number of examined biologically independent embryos (WT ES10 [n = 46]; WT ES13 [n = 94]; <sup>ΔM</sup>wunens<sup>-/-</sup> E59 [n = 34]; <sup>ΔM</sup>wunens<sup>-/-</sup> E510 [n = 35]; <sup>ΔM</sup> wunens<sup>-/-</sup> ES13 [n = 30]; <sup>ΔM</sup> wunens<sup>-/-</sup>, dnaseII<sup>-/-</sup> ES13 [n = 35]; <sup>ΔM</sup> wunens<sup>-/-</sup>, dnaseII<sup>-/-</sup> ES14 [n = 24]; <sup>ΔM</sup> wunens<sup>-/-</sup>, dnaseII<sup>-/-</sup> ES14 [n = 36]). See detailed description of the results in the main text. h–j p53 is cell autonomously required for PGC death. Representative images of a p53 mutant null embryo (h) and an embryo with PGC-specific p53 knockdown (i), both at ES 13, stained and presented as in Fig. 1b. PGCs (Vasa; red). Asterisks indicate gonadal PGCs. Arrowheads point at ectopically surviving PGCs. Scale bars, 50 µm. The corresponding quantifications of PGC death levels. All data points, including outliers, were presented in box plot format where the minimum is the lowest data point represented by the lower whisker bound, the maximum is the highest data point represented by the upper whisker bound, and the center is the median. The lower box bound is the median of the lower half of the dataset while the upper box bound is the median of the upper half of the dataset. Each dot corresponds to the number of PGCs in a single embryo to reflect n number, where n = number of examined biologically independent embryos. *p < 0.0001; **significantly different from WT; Student’s t-test, one-sided distribution. Note that some of the p53 mutant embryos contained more PGCs at ES 13 than in ES 10, implying an involvement of p53 in controlling cell division at these stages. k, l Nuclear translocation of DNase II is blocked in ectopically surviving p53 mutant (k) and knockdown (l) PGCs. Shown are ES 13 embryos stained to visualize the PGCs (Vasa; red), DNase II (green), and DNA (Hoechst, blue). The outlined areas (yellow squares) are magnified in the corresponding panels on the right. Asterisks indicate gonadal PGCs. Scale bars, 50 µm. Surviving midline PGCs are outlined (yellow circles). Note the non-nuclear DNase II localization in the surviving midline PGCs. Scale bars, 10 µm. The corresponding quantifications are presented in Supplementary Fig. 3i. Note that images of WT embryos stained with the anti-DNase II antibodies are shown in Fig. 4. m OE restores PGC death levels in p53 mutants. Shown is a representative image of a p53 mutant embryo at ES 13 with PGC-specific aif OE stained, and annotated as in (h, i). Asterisks indicate gonadal PGCs. Scale bars, 50 µm. The corresponding quantification is presented in (j). Note that OE of aif in an otherwise WT background does not affect PGC death levels, Fig. 7b.

Recent reports illuminate important crosstalk between the DDR and other checkpoint mechanisms critical for preventing genome instability, such as the spindle assembly checkpoint (SAC) and the DNA replication checkpoint<sup>33,54</sup>. Consistent with this idea, OE of two key components in these pathways, Orc2 (a component of the origin recognition complex [ORC]) and Bub3 (a main component of the SAC), both significantly attenuated PGC death (Supplementary Fig. 6a–c). Moreover, PGC-specific orc2 knockdown which did not affect PGC death levels, was sufficient to restore cell death of the ectopically surviving PGCs in the dnaseII<sup>lo</sup> mutant embryos, implying that activation of the DDR is sufficient to promote PGC death, even in the absence of DNA damage (Supplementary Fig. 6d).

Our genetic data supports a sequence of events, in which relocated nuclear DNase II elicits DNA damage, which in turn leads to DDR activation and cell death. To further test this model, we compromised the DDR in early embryos and monitored the localization of DNase II in the ectopically surviving PGCs. DNase II was detected in the nuclei of ectopically surviving PGCs, but not the gonadal PGCs, following either knockdown of chk1 or OE of Bub3, indicating that DDR activation is indeed downstream of DNase II nuclear translocation (Supplementary Fig. 6e–j). Of interest, the accumulation of DNase II in the nuclei of ectopically surviving PGCs was more pronounced following OE of Bub3 than knockdown of chk1, suggesting that Bub3 interferes with the DDR downstream of chk1, and that activation of the ATR/Chk1 branch of the DDR is required for further translocation of DNase II to the nucleus in a positive feedback loop (Supplementary Fig. 6g, j). Consistent with the latter idea, whereas WT dying midline PGCs at mid-to-late demolition stages readily displayed both nuclear DNase II and γH2Av staining, these were much rarer in the chk1 knockdown embryos (1 out of 50), displaying only partial nuclear localization of DNase II (Supplementary Fig. 4d, e).

AIF cooperates with DNase II to promote PGC death. It has been previously suggested that the mitochondrial protein AIF, which does not exert nuclease activity on its own, can nonetheless promote chromatin condensation and internucleosomal DNA fragmentation in vitro, a process sometimes referred to as chromatinolysis<sup>35</sup>. Furthermore, AIF has been implicated in...
some forms of caspase independent ACD pathways through associating with and facilitating the nuclear translocation and enhanced activity of different nucleases, including EndoG in *C. elegans* and LEI/LDNaseII and MIF in mammalian cells. Given the critical involvement of DNase II in eliciting DNA fragmentation and PGC death, we set up to explore whether AIF might also function to facilitate DNase II nuclear translocation and PGC death. Since homozygous mutant flies for a null allele of *aif* (*aifT52*) are embryonic lethal, we examined both *aif* hypomorphic mutants, carrying the null allele in trans to a weaker *aif* allele (*aifT2*), and PGC-specific *aif* knockdown embryos. Importantly, both the *aifT52/T2* maternally mutant embryos and the *aif* knockdown embryos displayed significant attenuation in PGC death, indicating that similar to DNase II, maternally supplied *aif* is cell autonomously required for PGC death (Fig. 7a, b).

We next explored possible genetic interactions between *aif* and *dnase II*. Since the *dnaseIIlo* allele encodes a weakly functional variant of DNase II, we first asked whether OE of AIF could compensate for the reduced DNase II activity in this genetic background. Notably, PGC-specific full-length AIF OE, which by itself did not increase PGC death levels in WT embryos, restored PGC death to almost normal levels in *dnaseIIlo* homozygous mutant embryos, as well as in *aif* knockdown embryos (which served as control), implying that AIF indeed cooperates with DNase II in mediating PGC death, and that PGC death is highly sensitive to the levels of both AIF and DNase II (Fig. 7b–d). Likewise, AIF OE restored PGC death to almost normal levels in
p53 knockdown embryos, linking between other components in the PGC death pathway and AIF (Fig. 3j, m).

The cooperation between AIF and DNase II was further examined by testing their ability to trigger non-apoptotic precocious PGC death. Taking advantage of the findings that, similar to AIF OE, PGC-specific DNase II OE could not trigger precocious PGC death in WT embryos on its own (although it could restore normal PGC death levels in dnaseII−/− mutant embryos [Supplementary Fig. 7a]), we tested the effect of double OE on PGC death. PGC-specific OE of both DNase II and AIF triggered precocious PGC death already at ES 10, such that some of the ES 13 embryos were almost completely devoid of PGCs (Supplementary Fig. 7b–d). It is noteworthy that despite the dramatic reduction in the pool of PGCs at ES 10, still only two-thirds of the remaining PGCs migrated towards the gonadal somatic precursor cells, indicating that induction of cell death by
double OE randomly occurs in the entire PGC population (Supplementary Fig. 7e). This is consistent with a recent report suggesting that the (midline) subset of PGCs that undergo cell death is already predetermined at a very early stage of PGC specification, according to the levels of germplasm they inherit from the oocyte, which in turn is determined by their spatial position at the posterior pole of the embryo. In contrast to Hid OE induced PGC apoptosis, AIF and DNase II OE induced PGC death was non-apoptotic, as the dying PGCs were TUNEL negative and cell death was not attenuated by OE of the apoptotic inhibitors, Diap1 and DroncDN. AIF can bind to DNase II and mediates its nuclear translocation. Given the involvement of AIF in the nuclear translocation of several nucleases, we next asked whether AIF might mediate PGC death by promoting the nuclear translocation of DNase II.

**AIF can bind to DNase II and mediates its nuclear translocation.** Given the involvement of AIF in the nuclear translocation of several nucleases, we next asked whether AIF might mediate PGC death by promoting the nuclear translocation of DNase II. Using the anti-DNase II antibodies to stain aif knockdown PGCs, revealed that most of the ectopically surviving midline PGCs in the affected embryos displayed non-nuclear DNase II (Fig. 7e–h).

**Fig. 6 Inactivation of the ATR/Chk1 branch of the DDR attenuates PGC death.** a Illustration of the two branches of the DDR pathway induced by DNA damage. Tested components for effects on PGC death are indicated in pink (for no effect) and green (for having an effect). Untested/unconfirmed components are in gray. Dashed lines indicate suggested interactions based on data from mammalian systems or when direct regulation was not observed in Drosophila. In brackets are the names of the Drosophila genes if different from the names of the mammalian homologs. Note that for the sake of simplicity, only parthanatos is mentioned as a process downstream of Chk1. b–f Representative images of ES 13 embryos with PGC-specific knockdowns of the indicated DDR pathway genes (corresponding to the cartoon model in a), stained and presented as in Fig. 1b. PGCs (Vasa; red). Dying PGCs are indicated by arrows; ectopically surviving PGCs by arrowheads; gonadal PGCs by asterisks. Whereas PGC death normally proceeded upon knockdown of components in the ATM/Chk2 branch of the DDR, knockdown of components in the ATR/Chk1 branch of the DDR attenuated PGC death (d–f). Scale bars, 50 μm. g Quantifications of PGC death levels in embryos corresponding to the genotypes in (b–f). All data points, including outliers, were presented in box plot format where the minimum is the lowest data point represented by the lower whisker bound, the maximum is the highest data point represented by the upper whisker bound, and the center is the median. The lower box bound is the median of the lower half of the dataset while the upper box bound is the median of the upper half of the dataset. Each dot corresponds to the number of PGCs in a single embryo to reflect number, where n = number of examined biologically independent embryos. ****p < 0.0001; NS, non-significant; Student's t-test, one-sided distribution. Added information about the red asterisks is in the main text.

**ARTICLE**

**NATURE COMMUNICATIONS | https://doi.org/10.1038/s41467-021-22622-1**

**NATURE COMMUNICATIONS** (2021) 12:2285 | https://doi.org/10.1038/s41467-021-22622-1 | www.nature.com/naturecommunications
recombinant glutathione S-transferase-tagged AIF (GST-AIF) and FLAG-tagged DNase II (FLAG-DNase II) in E. coli. FLAG pulldown analyses from cell lysates revealed that GST-AIF readily associated with FLAG-DNase II, but not with a control recombinant bacterial protein (FLAG-BrxA) or the anti-FLAG beads, indicating that DNase II can physically interact with AIF (Fig. 8a).

CypA binds to AIF and facilitates DNase II nuclear translocation. Nuclear translocation and the associated nuclease activity of AIF have been proposed to involve association with cyclophilin A (CypA)55,60, which similar to other cyclophilins, also contains a peptidyl-prolyl isomerase domain, which can affect protein folding of its associated partners. Searching the Drosophila UniProt for CypA-like protein sequences, revealed 4 different gene products with a cyclophilin-like domain, Cyp1, CG2852, CG17266, and Moca-cyp, sharing 77%, 59%, 57%, and 55% sequence identity with human CypA, respectively. PGC-specific knockdown of cyp1, CG2852, and CG17266, each attenuated PGC death in 60% of the embryos, suggesting an involvement and redundancy among at least three of the CypA-like proteins in PGC death (Fig. 8b, c). To investigate whether the cyclophilins might affect DNase II nuclear translocation, we stained cyp1 (the most similar cypA ortholog) knockdown embryos with anti-DNase II antibodies. Whereas in WT embryos 70% of the dying midline PGCs displayed nuclear DNase II localization, 100% of the ectopically surviving midline PGCs in the cyp1 knockdown embryos had no nuclear DNase II, suggesting that the cyclophilins affect PGC death by modulating (AIF-mediated) DNase II...
nuclear translocation (Fig. 8d, e). Interestingly, whereas PGC-specific OE of AIF in the cyp1 knockdown embryos restored PGC death in the affected embryos (Fig. 8c), 17% of these embryo displayed excessive PGC death (Fig. 8f), suggesting that the balance between the levels of AIF and the cyclophilins may modulate the cell death promoting activity of AIF. To test whether Cyp1 can indeed physically associate with AIF, full-length recombinant Twin-Strep-tagged Cyp1 (Cyp1-Twin-Strep) and GST-AIF were co-expressed in E. coli. Cell lysates used in a Strep-Tactin pull-down analyses showed that whereas Strep-Tacin resin alone could not pull down GST-AIF, the latter was readily co-pulled down with Cyp1-Twin-Strep, demonstrating that Cyp1 can bind to AIF in vitro (Fig. 8g).

The DNA damage sensor PARP-1 mediates PGC death. Previous reports have implicated AIF as a key mediator of a related group of non-apoptotic cell death subtypes, commonly termed parthanatos (PAR for Poly(ADP-ribose) and Thanatos, the personification of death in the Greek mythology). According to the current model, parthanatos is triggered by OE of the DNA damage sensor, Poly(ADP-ribose) polymerase-1 (PARP-1), or its activation following DNA damage caused by genotoxic stress or excitotoxicity, and is manifested by extensive DNA damage. Given the striking anatomical and molecular similarities between PGC death and parthanatos, we hypothesized that PGC death might constitute a developmental form of this cell death pathway. To address this idea, we first examined whether PARP-1 might also be involved in PGC death. As opposed to mammalian cells, Drosophila contains only a single PARP enzyme, Parp1. Critically, PGC-specific parp1 knockdown attenuated PGC death in 60% of the examined embryos, suggesting a requirement for PARP-1-like activity in PGC death (Fig. 7i, j).

Following recruitment to diverse types of DNA lesions, PARP-1 undergoes conformation shift and activation, promoting the synthesis and attachment of PAR chains to a variety of proteins, hence altering their conformation and structure, as well as facilitating interactions with other proteins. This post-translational modification process is known to regulate a wide variety of cellular processes, including initiation of the DDR and DNA repair pathways. To visualize PARP-1 activity during PGC death, we stained ES 10 embryos with anti-PAR antibodies. Nuclear accumulation of PAR was detected in numerous cells in the embryo, with particularly strong expression in some of the midline PGCs (Supplementary Fig. 8a). PGC-specific parp1 knockdown significantly reduced the PAR signal in these cells, suggesting that most of the PGC PARs are produced by Parp1 (Supplementary Fig. 8b). Therefore, consistent with the genetic data, PARP-1 activity is present in PGCs during the relevant stages of PGC death.

The release of a subset of the AIF protein from the mitochondria and/or its translocation to the nucleus was reported to involve covalent binding of PAR polymers. We therefore reasoned that at least some of the PAR polymers might be detected in the cytoplasmic compartment during PGC death. However, although the prominent nuclear PAR signal disappears during advanced PGC death stages in about 80% of the midline PGCs while remaining nuclear in 100% of the gonadal PGCs, we could not visualize cytoplasmic accumulation of PAR in the dying midline PGCs (Fig. 9a–d). One explanation for this failure could be that the phase of PGC death involving cytoplasmic PAR activity is very short. To test this idea, we sought to reduce the rate of PGC death by compromising (but not blocking) this ACD pathway. Intriguingly, visualizing PAR in the PGC-specific chkl knockdown embryos, where PGC death is attenuated (Fig. 6f, g), revealed ectopically surviving midline PGCs halted at various steps during the release of the PAR polymers to the cytoplasm. Whereas in some of the midline PGCs PAR was still confined to the nucleus (Fig. 9e, g), other PGCs contained both nuclear and cytoplasmic PAR (Fig. 9e, h), while a third PGC population displayed PAR exclusively in the cytoplasm (Fig. 9e, i). As expected, in the gonadal PGCs, PAR exclusively resided in the nucleus (Fig. 9e, f). Taken together, these observations are in line with the parthanatos model in which nuclear PAR generated by PARP-1 is released and signals in the cytoplasm.

DNase II and PARP-1 engage in positive amplification loop. Our results thus far support a model in which PGC death is mediated by DNase II-induced DNA damage and by PARP-1-dependent activation of the DDR (see the cartoon model in Fig. 10). To investigate the inter-regulation between these two modalities in PGC death, we monitored PARP-1 activity (the presence of PAR polymers) in PGCs of dnaselO mutant embryos, as well as the nuclear translocation of DNase II in the background of PGC-specific parp1 knockdown. In contrast to the dying PGCs in WT embryos, in which an intense nuclear PAR signal...
disappears during advanced PGC death stages (Fig. 9a-c), ectopically surviving midline PGCs in the \textit{dnaseIIlo} mutants displayed persistent nuclear PAR signal, suggesting that the release of the PAR polymers to the cytoplasm requires DNase II activity (Supplementary Fig. 8c-e). Conversely, when \textit{parp1} was knocked down, the translocation of DNase II to the nucleus in the ectopically surviving midline PGCs was significantly reduced (detected only in two PGCs within two different embryos out of 50 examined embryos), suggesting that PARP-1 activity modulates DNase II nuclear translocation (Supplementary Fig. 8f-i). Given the synergistic interaction between AIF and DNase II, and that AIF modulates the nuclear translocation of DNase II during PGC death, as well as the idea that PARP-1 activity (cytoplasmic PAR) promotes the release of AIF from the mitochondria and translocation to the nucleus\textsuperscript{26}, these observations suggest that PARP-1 and DNase II engage in a positive feedback amplification loop, leading to activation of the DDR and consequent PGC death by parthanatos.

**Discussion**

In this study, we discovered a developmental form of the ACD pathway called parthanatos, by which PGCs undergo cell death during development, demonstrating that parthanatos is not limited to stress or pathological conditions. Our collective results support a model in which developmental parthanatos is triggered by lysosomal components and is mediated by the DDR. At the center of this pathway is a positive feedback amplification loop involving PARP-1 and DNase II, which through PAR and AIF mediators, enhances PARP-1 activation and DNase II nuclear translocation, respectively,
citing in the activation of the ATR/Chk1 branch of the DDR pathway and consequent cell death (Fig. 10). This model significantly expands the current knowledge about parthanatos, as it implicates both lysosomal components, in particular DNase II, and the DDR in the activation and execution of this ACD pathway, respectively. Furthermore, contrary to the notion that DNA fragmentation/damage constitutes the final/execution stage of parthanatos, the current study demonstrates that activation of the DDR pathway downstream of DNA damage is essential for the execution of this ACD pathway.

Molecularly, parthanatos is mediated and hence defined by the action of 3 critical components: PARP-1, AIF, and a PARP-1-dependent AIF-associated nuclease (PAAN)\(^{27,28,65}\). Although additional comparative studies of the current parthanatos paradigms, as well as of yet unidentified parthanatos subtypes, are still required in order to reliably generalize a common mechanism, it appears that PARP-1 and AIF are invariably required for triggering and mediating parthanatos, while the identity of the PAAN could vary between different systems and cell types. Whereas in the current work we identified DNase II as the AIF interacting nuclease critical for PGC death, a recent study in mammalian cells revealed MIF, which does not have a Drosophila homolog, to be the critical PAAAs.\(^{27}\). In addition, AIF was shown to interact with other nucleases, such as EndoG and LEI/LDNaseII, promoting apoptosis in C. elegans and triggering caspase-independent cell death (presumably parthanatos) in mammalian cells, respectively.\(^{57,66}\) We therefore propose that the exact identity of the nuclease involved might serve as an initial basis for classification of the different parthanatos subtypes. Furthermore, the remarkable anatomical and molecular conservations between PGC death (DNase II–parthanatos) and MIF–parthanatos might also imply that, similar to DNase II–parthanatos, mediation and execution of other parthanatos subtypes may also involve the DDR.

Our findings that inactivation of DNase II partially rescues PGC death induced by the lack of maternal wunens, place DNase II–parthanatos pathway downstream of Wunens in the PGCs. How the Wunens trigger PGC death is unclear, but it was suggested that the balance between the soma and germline expressed Wunens controls PGC survival and death, by competing for the hydrolysis of an extracellular lipid phosphatidylserine.\(^{36,37}\) Interestingly, Wun2 was shown to also promote internalization and rapid cytoplasmic accumulation of the dephosphorylated lipid substrates.\(^{36}\) Given that chronic lipid overload can promote lysosome dysfunction and LMP, a conceivable hypothesis would be that the induction of midline PGC death might occur by Wunens-dependent lipotoxicity-induced LMP.

In conclusion, cell death of the aberrantly migrating cells are evolutionary conserved features of PGCs.\(^{34}\) Intriguingly, PGC death in mice might also diverge from canonical apoptosis: although mouse embryos lacking Bax, the proapoptotic Bcl-2 family member, display a delay in PGC death, the ectopically surviving PGCs still die via a Bax-independent mechanism later in development, implying the involvement of an ACD pathway.\(^{68}\) Furthermore, a caspase-independent role of Bax in lipid-induced LMP was also noted, suggesting that similar mechanisms might operate in both Drosophila and mouse PGCs to trigger cell death. Future comparative studies of PGC death in Drosophila and mammals, as well as of other parthanatos paradigms, will improve insight into the signaling pathways and mechanisms underlying this ACD pathway, and will shed light on the molecular commonalities and differences between the different parthanatos subtypes and their possible significance. Since cell death by parthanatos has been implicated in the pathogenesis of many important human diseases, and because targeted induction of parthanatos could overcome the inherent resistance of many cancer cells to (apoptotic) cell death, the importance of addressing these questions is of considerable significance for translational research and applications.

**Methods**

**Drosophila strains and crosses.** Flies were raised on standard yeast/molasses medium at 25 °C. The following stocks were used: Oregon R and nos-Gal4-VP16 (on 3rd chromosome; Bloomington Drosophila Stock Center [BDSC], stock #4937; was used in all the relevant experiments except for the ones mentioned above); UAS-p35 and UAS-hid7 from...
Fig. 9 PAR polymers are released to the cytoplasm during PGC death. a–d Dying PGCs display no PAR polymers in the nucleus. A representative image of the midline region of an ES 10 WT embryo stained to visualize PAR (anti-PAR; green), PGCs (Vasa; red), and DNA (Hoechst; blue) (a), and magnifications of the areas outlined by yellow rectangles (b, c). Arrows are pointing at two dying PGCs with highly reduced levels of nuclear PAR. Note that the PAR polymers are predominantly localized in the nucleus of living (intact) PGCs, but are almost absent in dying PGCs (a condensed dying PGC is circled). Scale bars, 10 μm. Quantification of the percentage of gonadal and midline PGCs devoid of PAR signal or with nuclear PAR in WT ES 11 embryos (d). Green column outline indicates intact living cells, while red column outline indicates condensed and distorted dying PGCs. n number is shown in brackets where n = number of examined PGCs.  

e–i Ectopically surviving PGCs due to compromised DDR reveal the release of the PAR polymers from the nucleus to the cytoplasm. Shown is a representative image of an ES 13 embryo with PGC-specific chk1 knockdown (e) stained as in (a). An asterisk indicates gonadal PGCs. Magnifications of several areas outlined by yellow rectangles in (e) are presented in (f–i). Shown are gonadal PGCs (f) and an ectopically surviving midline PGC (g) predominantly displaying PAR in the nucleus (circled), an ectopically surviving midline PGC displaying both nuclear and cytoplasmic PAR (h, circled), and ectopically surviving midline PGCs displaying only cytoplasmic PAR (i, circled). Scale bars in e 50 μm, f–i 10 μm.
AIF and PAR, respectively), activates the ATR/Chk1 branch of the DDR massive release of AIF from the mitochondria, respectively. This positive proteins both in and outside the nucleus, activating the DDR and promoting RNAi Center (VDRC):

lysosomes might promote the initial release of AIF from the mitochondria midline experience high somatic Wunens (LPP) activity, which may induce to another, while black arrows indicate signaling. PGCs that remain in the enlargement of a dying PGC (right), featuring the molecular model of PGC Fig. 10 An integrated model of the developmental form of PGC death by parthanatos. An illustration of an embryo at ES 13 (left) and an enlargement of a dying PGC (right), featuring the molecular model of PGC death. Purple arrows indicate translocations from one cellular compartment to another, while black arrows indicate signaling. PGCs that remain in the midline experience high somatic Wunens (LPP) activity, which may induce LMP and the release of lysosomal hydrolases, including DNase II, Cathe B and Cath. (See the Discussion for more details about a possible link between the Wunens and LMP). The release of Cathe B from the lysosomes might promote the initial release of AIF from the mitochondria as was reported (dashed arrow). AIF then, together with CypA, promotes the translocation of DNase II to the nucleus, presumably as a CypA-AIF-DNase II complex. In the nucleus, DNase II cleaves the DNA, leading to PARP-1 activation, which in turn generates PAR. PAR modifies proteins both in and outside the nucleus, activating the DDR and promoting massive release of AIF from the mitochondria, respectively. This positive feedback amplification loop between DNase II and PARP-1 (mediated by AIF and PAR, respectively) activates the ATR/Chk1 branch of the DDR (presumably to a critical threshold), ultimately leading to PGC death.

Antibodies. The primary antibodies used in this study were polyclonal rabbit anti-Vasa (1:250, sc-30210; Santa Cruz), mouse anti-PAR (1:20, ab14459; Abcam), rabbit anti-cleaved Dcp-1 (1:100, 9578; Cell Signaling), rabbit anti-Hb9 (1:100; from J. Skeath, Haverford College, PA), and the following antibodies from the Hybridoma Bank (DSHB): polyclonal rabbit anti-Arl8 (1:20) and monoclonal mouse anti-p53 (direct, 46F11) and mouse anti-p53 (direct, UNO93-5-2.1).

Anti-DNase II antibodies were generated as follows: GST-tagged DNase II protein without the signal peptide (spanning amino acids 1-19) was expressed in E. coli. A Coomassie Blue-stained gel slice of the corresponding size was used as an antigen to generate polyclonal antibody in two rats. One immunization and four boosts were done for each animal and after the 4th boost 60 ml serum were obtained. The antibody was used at a dilution of 1:20.

TUNEL and Top I-mediated ligation assay. Embryos were staged, collected, and fixed as described above. TUNEL labeling was carried out using the ApoTag Red In Situ Apoptosis Detection Kit (Millipore, Billerica, MA, USA). Fixed embryos were washed in 1x PBS + 0.1% Tween 20 (PBTw), washed in equilibration buffer for one hour at room temperature, incubated in TdT reaction mix (70% reaction buffer to 30% TdT enzyme) for three hours at 37 °C, and then incubated in stop buffer for four hours at 37 °C. Next, embryos were blocked in BTN solution (1xBS, 0.3% Triton X-100, and 5% normal goat serum) for one hour at room temperature, washed in 1xBS, incubated in anti-dioxigenin and primary antibody overnight at 4 °C, washed with 1xBSS, incubated for two hours at room temperature, washed, mounted, and visualized as described above.

Quantiﬁcation of PGC death. The number of Vasa-positive PGCs in each embryo was manually counted following confocal microscopy imaging. For each genotype, the average PGC number at ES 10 was set as 1 and the individual values at ES 10 and 13 were expressed as a percentage of the average value at ES 10. The values were then plotted using box and whisker charts, in which the median is shown as a line in the middle of the box and the minimum and maximum values are the bottom and the top of the whiskers respectively. Each dot in the box represents a single embryo clearly reflecting the sample number (n). On average, a sample number of 23 was used for each genotype. Note, whereas at ES 13 most of the dead PGCs are already eliminated, dying PGCs can be sometimes still detected by virtue of their highly condensed morphology and relatively faint Vasa staining signal, and are omitted from the counts. PGC death index in Fig. 1d was calculated as follows: The number of embryos with at least one dying PGC (condensed/distorted) was divided by the total number of examined embryos giving a value A. The total number of dying PGCs in all the embryos was divided by the number of embryos with at least one dying PGC, giving a value B. Death index equals AxB.
gift from Rotem Sorek, WIS) genes were sub-cloned into the 1st ORF of the expression vector pACYCDuet-1 (Novagen), including an N-terminal FLAG-tag followed by a TEV cleavage site. Full-length Drosophila ORF of cyclin B (CyB; cDNA clone R62690) was sub-cloned into the 2nd ORF of pACYCDuet-1, including a C-terminal Twin-Strep-tag.

Protein expression: Individual protein expression and co-expression studies were performed in E. coli BL21 (DE3) cells. Expression was performed in LB medium supplemented with the appropriate antibiotics (Kanamycin and/or chloramphenicol). Expression was induced with 200 μM IPTG followed by shaking at 15°C for ~16 h. Cell pellets were stored at -20°C before processing.

Protein pulldown: For FLAG-tag pulldown, cells were lysed by sonication in Tri-buffered saline (TBS) buffer supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 μl/mL of protease inhibitor cocktail (Set IV, EMD Chemicals, Inc.). Protein pulldown experiments were performed using Anti-Flag-tag [HRP] mAb (GenScript # A01428-100) and mouse Anti-GST [HRP] mAb (GenScript #A00866-100). Dilution used for all antibodies was 1:1000.

For Strep-tag pulldown cells were lysed by sonication in 50 mM Tris-HCl, pH 8, 500 mM NaCl buffer supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 μl/mL of protease inhibitor cocktail (Set IV, EMD Chemicals, Inc.). Protein pull-down experiments were performed using Strep-Tactin Sepharose resin (#-12010, IBA) according to the manufacturers’ recommendations. Western blotting was performed using THE™ NWSHPQFKEQ Tag Antibody [HRP], mAb, Mouse (GenScript #A01742-40) and mouse Anti-GST [HRP] mAb (GenScript #A00866-100). Dilution used for all antibodies was 1:1000.

Validation of knockdown and overexpression flow lines: Western blotting: For FLAG; testes were dissected from young adult WT and y lines that were readily available from the authors. Source data are provided with this paper.

Received: 26 March 2020; Accepted: 23 March 2021; Published online: 16 April 2021

Validation of knockdown and overexpression flow lines

Western blotting: For FLAG; testes were dissected from young adult WT and y lines that were readily available from the authors. Source data are provided with this paper.

Received: 26 March 2020; Accepted: 23 March 2021; Published online: 16 April 2021

Validation of knockdown and overexpression flow lines

Western blotting: For FLAG; testes were dissected from young adult WT and y lines that were readily available from the authors. Source data are provided with this paper.

Received: 26 March 2020; Accepted: 23 March 2021; Published online: 16 April 2021
22. Mondragon, A. A. et al. Lysosomal machinery drives extracellular acidification to direct non-apoptotic cell death. Cell Rep. 27, 11–19.e3 (2019).
23. Dawid, K. K., Anbali, S. A., Dawson, T. M. & Dawson, V. L. Parthanatos, a messenger of death. Front. Biosci. (Landmark Ed.) 14, 1116–1128 (2009).
24. Fatokun, A. A., Dawson, V. L. & Dawson, T. M. Parthanatos: mitochondrial-linked mechanisms and therapeutic opportunities. Br. J. Pharmacol. 171, 2000–2016 (2014).
25. Wang, X. & P. Parthanatos in the pathogenesis of nervous system diseases. Neuroscience 449, 241–250 (2020).
26. Wang, Y. et al. Poly(ADP-ribose) (PAR) binding to apoptosis-inducing factor is critical for PAR polymerase-1-dependent cell death (parthanatos). Sci. Signal. 4, ra20 (2011).
27. Wang, Y. et al. A nuclease that mediates cell death induced by DNA damage and poly(ADP-ribose) polymerase-1. Science 354, 6842 (2016).
28. Yu, S. W. et al. Mediation of poly(ADP-ribose) polymerase-1-dependent cell death by apoptosis-inducing factor. Science 297, 259–263 (2002).
29. Fan, J., Dawson, T. M. & Dawson, V. L. Cell Death Mechanisms of Neurodegeneration. Adv. Neurobiol. 15, 403–425 (2017).
30. Baxter, F., Chen, Y., Xu, Y. & Swanson, R. A. Mitochondrial dysfunction induced by nuclear poly(ADP-ribose) polymerase-1: a treatable cause of cell death in stroke. Transl. Stroke Res. 5, 136–144 (2014).
31. van Wijk, S. J. L. & Hageman, G. J. Poly(ADP-ribose) polymerase-1 mediated casapse-independent cell death after ischemia/reperfusion. Free Radic. Biol. Med. 39, 81–90 (2005).
32. Cinalli, R. M., Rangan, P. & Lehmann, R. Germ cells are forever. Curr. Biol. 27, 559–562 (2008).
33. Starz-Gaiano, M., Cho, N. K., Forbes, A. & Lehmann, R. Spatially restricted DNA repair and chromatin remodelling. J. Neurosci. 137, 128–137 (2013).
34. Richardson, B. E. & Lehmann, R. Mechanisms guiding primordial germ cell migration: strategies from different organisms. Adv. Neurobiol. 15, 453–464 (2017).
35. Sano, H., Renault, A. D. & Lehmann, R. Control of lateral migration and germ cell elimination by the Drosophila melanogaster lipid phosphate phosphatases Wunen and Wunen 2. J. Cell Biol. 171, 675–683 (2005).
36. Phenotypic differences in a single maternal factor determine survival probabilities among Drosophila germ cells. Curr. Biol. 27, 291–297 (2017).
37. Slaidda, M. & Lehmann, R. DNA repair and chromatinolysis. J. Cell Sci. 128, 1151–1157 (2015).
38. Slaidina, M. & Lehmann, R. Quantitative differences in a single maternal factor determine survival probabilities among Drosophila germ cells. Curr. Biol. 27, 291–297 (2017).
39. Yamada, Y., Davis, K. D. & Coffman, C. R. Programmed cell death of primordial germ cells in Drosophila is regulated by p53 and the Outsiders monooxynasate transporter. Development 135, 207–216 (2008).
40. Leprêtre, C., Tchakarska, G., Blibech, H., Lebon, C. & Torriglia, A. Apoptosis-inducing factor (AIF) and leukocyte elastase inhibitor/DNase II (LEL/DNaseII), can interact to conduct caspase-independent cell death. Apoptosis 18, 1048–1059 (2013).
41. Joza, N. et al. Essential role of the mitochondrial apoptosis-inducing factor in programed cell death. Nature 410, 549–554 (2001).
42. Mukae, N., Yokoyama, H., Yokokura, T., Sakoyama, Y. & Nagata, S. Activation of the innate immune response in Drosophila by endogenous chromosomal DNA that escaped apoptotic degradation. Genes Dev. 16, 2662–2671 (2002).
43. Candé, C. et al. AIF and cyclophilin A cooperate in apoptosis-associated chromatinolysis. Oncogene 23, 1514–1521 (2004).
44. Zhang, J., Dawson, V. L., Dawson, T. M. & Snyder, S. H. Nitric oxide activation of poly(ADP-ribose) synthetase in neurotoxicity. Science 263, 686–689 (1994).
45. Miwa, M., Hanai, S., Poltronieri, P., Uchida, M. & Uchida, K. Functional analysis of poly(ADP-ribose) polymerase in Drosophila melanogaster. Mol. Cell. Biochem. 193, 103–107 (1999).
46. Langelier, M. F., Eisemann, T., Riccio, A. A. & Pascal, J. M. PARP family enzymes: regulation and catalysis of the poly(ADP-ribose) posttranslational modification. Curr. Opin. Struct. Biol. 53, 187–198 (2018).
47. Ray Chaudhuri, A. & Nussenzweig, A. The multifaceted roles of PARP1 in DNA repair and chromatin remodelling. Nat. Rev. Mol. Cell Biol. 18, 610–621 (2017).
48. Mandir, A. S. et al. NMDA but not non-NMDA excitotoxicity is mediated by poly(ADP-ribose) polymerase. J. Neurosci. 20, 8005–8011 (2000).
49. Wang, X., Yang, C., Chai, J., Shi, Y. & Xue, D. Mechanisms of AIF-mediated apoptotic DNA degradation in Caenorhabditis elegans. Science. 298, 1587–1592 (2002).
50. Iwata, Y., Fujii, T., Ando, S., Nakashima, N., Sugimoto, S., Yamasaki, Y., Maekawa, T., Shida, K. & Masuda, K. Phosphorylation of p53 by Akt promotes neuronal health, stress tolerance, and longevity but is dispensable for mammalian neuronal survival. Aging Cell 20, 1–15 (2021).
51. Maggett, K. A. & Golic, K. G. Highly efficient sex chromosome interchanges produced by I-Crel expression in Drosophila. Genetics 171, 1103–1114 (2005).
52. Smith, J. H., MacPhee, D. A. & Thirumal-Chakravarthi pathways in DNA damage signaling and cancer. Adv. Cancer Res. 108, 73–112 (2010).
53. Sulli, G., Di Micco, R. & Di Fagagna, F. D. A. Crostalk between somatotrophic cell state and DNA damage response in cellular senescence and cancer. Nat. Rev. Cancer 12, 709–720 (2012).
54. Lawrence, K. S. & Enger, J. P. Spindle checkpoint: More than just keeping track of the spindle. Trends Cell Mol. Biol. 10, 141–150 (2015).
55. Candé, C., Vahsen, N., Garrido, C. & Kroemer, G. Apoptosis-inducing factor and poly(ADP-ribose) polymerase-1 are dispensable for a caspase-independent after all Cell Death Differ. 11, 591–595 (2004).
78. Scott, R. C., Juhasz, G. & Neufeld, T. P. Direct induction of autophagy by Atg1 inhibits cell growth and induces apoptotic cell death. *Curr. Biol.* 17, 1–11 (2007).

79. Tain, L. S. et al. Drosophila HtrA2 is dispensable for apoptosis but acts downstream of PINK1 independently from Parkin. *Cell Death Differ.* 16, 1118–1125 (2009).

80. Deluca, S. Z. & O’Farrell, P. H. Barriers to male transmission of mitochondrial DNA in Drosophila melanogaster. *EMBO J.* 16, 634 (2016).

81. Xiao, C., Mileva-Seitz, V., Seroude, L. & Robertson, R. M. Targeting HSP70 to motoneurons protects locomotor activity from hyperthermia in Drosophila. *Dev. Neurobiol.* 67, 438–455 (2007).

82. Rong, Y. S. et al. Targeted mutagenesis by homologous recombination in *D. melanogaster*. *Dev. Cell* 29, 233–247 (2019).

83. Okada, H., Yagi, R., Gardeux, V., Deplancke, B. & Hafen, E. Sex-dependent and sex-independent regulatory systems of size variation in natural populations. *Mol. Syst. Biol.* 15, e9012 (2019).

84. Kuleshova, Y., Puah, W. C. & Wasser, M. Live imaging of muscle histolysis in *Drosophila* using live cell microscopy. *Biochim. Biophys. Acta* 1864, 728–738 (2016).

85. Na, H. jin, Akan, I., Abramowitz, L. K. & Hanover, J. A. Nutrient-driven O-GlcNAcylation controls DNA damage repair signaling and stem/progenitor cell homeostasis. *Cell Rep.* 31, 107632 (2020).

86. Polit, Y. et al. Paternal mitochondrial destruction after fertilization is inhibited by a common endocytic and autophagic pathway in *Drosophila*. *Dev. Cell* 29, 305–320 (2014).

87. Bills, V. et al. Developmentally regulated autophagy is required for eye formation in *Drosophila*. *Autophagy* 14, 1499–1519 (2018).

88. Fujisawa, Y., Kosakamoto, H., Chihara, T. & Miura, M. Non-apoptotic function of drosophila caspase activation in epithelial thorax closure and wound healing. *Dev. Biol.* 416, 169037 (2019).

89. Schwartz, R., Guichard, A., Franc, N. C., Roy, S. & Bier, E. A Drosophila Model for Clostridium difficile Toxin CDT Reveals Interactions with Multiple Effector Pathways. *Science* 23, 100865 (2020).

90. Shinoda, N., Hanawa, N., Chihara, T., Koto, A. & Miura, M. Dronic-independent basal executioner caspase activity sustains Drosophila imaginal tissue growth. *Proc. Natl Acad. Sci. USA* 116, 20539–20544 (2019).

91. Unger, T., Jacobovitch, Y., Dantes, A., Bernheim, R. & Peleg, Y. Applications of the Restriction Free (RF) cloning procedure for molecular manipulations and protein expression. *J. Struct. Biol.* 172, 34–44 (2010).

92. Peleg, Y. & Unger, T. Application of high-throughput methodologies to the expression of recombinant proteins in E. coli. *Methods Mol. Biol.* 426, 197–208 (2008).

93. Livak, K. J. & Schmittgen, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT method. *Methods* 25, 402–408 (2001).

94. Hu, Y. et al. FlyPrimerBank: an online database for *Drosophila melanogaster* gene expression analysis and knockdown evaluation of RNAi reagents. *G3 (Bethesda)* 3, 1607–1616 (2013).

Acknowledgements
We are grateful to Lilač Gilboa, Hyung Don Ryoo, Hermann Steller, Bruno Lemaître, Joseph Penninger, Eric Baehrecke, Jim Skeath, Akira Nakamura, the Vienna *Drosophila* RNAi Center (YDRC), the *Drosophila* RNAi Screening Center (DRSC) and Transgenic RNAi Project (TRIP), and the Bloomington *Drosophila* Stock Center for providing additional stocks and reagents. We thank the Arama Laboratory members for their encouragement and advice. We note Asa Tiroski and Dr. Shira Albeck from the Israel Structural Proteomics Center (ISPc) at the WIS for helping to carry out the co-immunoprecipitation experiments. We warmly thank Genia Brodsky from the WIS Graphic Design Department for help with the graphic illustrations. We warmly thank Eyal Schechter for his excellent comments on the manuscript. This research was supported by grants from the European Research Council under the European Union’s Seventh Framework Programme (FP/2007-2013)/ERC grant agreement (616088), the ISRAEL SCIENCE FOUNDATION (grant No. 1279/19), and the Minerva Foundation with funding from the Federal German Ministry for Education and Research. E.A. is supported by a research grant from the Estate of Emile Mimran. E.A. is the Incumbent of the Harry Kay Professional Chair of Cancer Research.

Author contributions
E.A. and LT-I. designed the project and analyzed all experiments. Execution of the experiments was carried out by all the authors. E.A. and LT-I. wrote the manuscript. All authors discussed the results and commented on the manuscript.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41467-021-22622-1.

Correspondence and requests for materials should be addressed to E.A.

Peer review information Nature Communications thanks the anonymous reviewers for their contributions to the peer review of this work. Peer review reports are available.

Reprints and permission information is available at http://www.nature.com/reprints

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

© The Author(s) 2021