AMPK blocks starvation-inducible transgenerational defects in *Caenorhabditis elegans*

Emilie Demoinet[a,b], Shaolin Li[a], and Richard Roy[a,1]

[a]Department of Biology, McGill University, Montreal, QC, Canada H3A 1B1; and [b]Institute of Biology Valrose (iBV), CNRS, INSERM, Université Nice Sophia Antipolis, 06100 Nice, France

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Life history events, such as traumatic stress, illness, or starvation, can influence us through molecular changes that are recorded in a pattern of characteristic chromatin modifications. These modifications are often associated with adaptive adjustments in gene expression that can persist throughout the lifetime of the organism, or even span multiple generations. Although these adaptations may confer some selective advantage, if they are not appropriately regulated they can also be maladaptive in a context-dependent manner. We show here that during periods of acute starvation in *Caenorhabditis elegans* larvae, the master metabolic regulator AMP-activated protein kinase (AMPK) plays a critical role in blocking modifications to the chromatin landscape. This ensures that gene expression remains inactive in the germ-line precursors during adverse conditions. In its absence, critical chromatin modifications occur in the primordial germ cells (PGCs) of emergent starved L1 larvae that correlate with compromised reproductive fitness of the generation that experienced the stress, but also in the subsequent generations that never experienced the initial event. Our findings suggest that AMPK regulates the activity of the chromatin modifying COMPASS complex (complex proteins associated with Set1) to ensure that chromatin marks are not established until nutrient/energy contingencies are satisfied. Our study provides molecular insight that links metabolic adaptation to transgenerational epigenetic modification in response to acute periods of starvation.

epigenetics | AMPK | histone methyltransferase | COMPASS | *C. elegans*

In the winter of 1944/1945, the Nazi regime blockaded regions of Holland and rationed food for all inhabitants without exception, including pregnant women and newborn children. The infamous "Hongerwinter" came to an end in the spring of 1945, with the arrival of the allied forces providing supplies and medical help for the survivors. Although birth weight and size of the Hongerwinter infants varied with the period of gestation that occurred during the famine, surprisingly few developmental anomalies were immediately observed despite the extreme mal/undernutrition of the mothers (1–3). However, years afterward, the same individuals showed an abnormally elevated frequency of obesity, cardiac disease, and even schizophrenia, despite the lack of genetic history of these diseases. Further analysis suggested that DNA methylation patterns in specific regions of the genome were altered in many of the Hongerwinter children (1, 4).

Although largely correlative, the study of the children born during and after the Hongerwinter, along with other studies of famine victims, provide compelling evidence to support a developmental origin of a broad spectrum of disorders. Some of these disorders may rely on epigenetic changes that alter gene expression downstream of a single event or prolonged environmental conditions (5, 6). At present it has proven difficult to link the observed epigenetic changes to any obvious adaptive response to the nutrient stress, or how such a response may underlie the clinical manifestations that arise in individuals several years after the initial stress. Moreover, the nature of such changes may potentially extend beyond the described changes in DNA methylation to impinge upon chromatin marks that could heritably modify gene expression in a DNA sequence-independent manner.

Although *Caenorhabditis elegans* does not possess the enzymes involved in cytosine methylation, it does exhibit various forms of epigenetic inheritance. These epigenetic traits are mediated largely through chromatin modifications and a heritable population of small RNAs that collectively dictate whether regions of the genome are expressed or silenced (7–9). Recently, it was shown that the composition of a large repertoire of RNAs is sensitive to starvation during the first larval (L1) diapause, and could reflect some of the adaptive changes in gene expression that are transmitted in subsequent generations (10). Furthermore, starvation during early stages of development can induce long-term phenotypic consequences for exposed animals and increase stress resistance in their descendants (11). These data therefore suggest that in *C. elegans* early life-history events can exert a lasting transgenerational impact associated with an adaptive response to the initial event.

We show here that the AMP-activated protein kinase (AMPK) is required not only to adjust to the stress associated with starvation during the L1 stage in *C. elegans*, but also to block epigenetic modifications typical of gene activation during this period of acute starvation. In its absence, the histone methyltransferase-containing COMPASS complex (complex proteins associated with Set1) becomes misregulated, generating chromatin modifications that have detrimental effects on reproductive fitness, not only in the generation of animals that experienced the starvation, but in subsequent generations that were never starved.

Results

Following embryogenesis, emergent *C. elegans* L1-stage larvae remain in a nondeveloping, diapause-like state until they encounter a nutrient/energy source that will trigger the onset of the post-embryonic developmental program (12, 13). Whereas wild-type

Significance

Following periods of famine, a reproducible spectrum of disorders often manifest long after the period of starvation. Curiously, many of these diseases arise in individuals that have no apparent genetic history of the disorder, although they do correlate with specific epigenetic modifications. We used *Caenorhabditis elegans* as a model to understand how acute periods of starvation might result in physiological or developmental consequences in a single generation or over multiple generations following the initial period of stress. Our data suggest that the AMP-activated protein kinase, an enzyme that mediates metabolic adjustment during starvation, is required to block germ-line gene expression during these conditions. In its absence the inappropriate activation of germ-line transcription results in sterility and transgenerational reproductive defects.

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[1]To whom correspondence should be addressed. Email: richard.roy@mcgill.ca.

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L1 larvae can survive up to 2 wk in this starved state, loss of activity in the metabolic regulatory kinase AMPK/*aak-1/2*, the tumor suppressor ortholog phosphatase and tensin homolog deleted on chromosome 10 (PTEN)/*daf-18,* or the histone deacetylase 1 ortholog *hda-1*, sensitizes animals to this stress, causing them to die prematurely en masse (Fig. S1*A*) (13–16). Although it is less clear how the other two genes affect survival of starved L1 larvae, AMPK becomes activated in response to starvation and phosphorylates diverse substrates to facilitate metabolic adjustment (17). These targets include key metabolic enzymes, whereas AMPK activation has also been linked to changes in gene expression through chromatin modification (18). To determine whether AMPK may establish or maintain changes within the chromatin landscape downstream of acute starvation, we examined the role of AMPK during the metabolic adaptation associated with the L1 diapause in *C. elegans*.

In many organisms the loss of AMPK is lethal (19, 20); however, *C. elegans* mutants that have no AMPK signaling because of the disruption of its two catalytic subunits (AMPK/*aak-1/2*) are viable but exhibit defects during periods of nutrient/energy stress. This is most notable during the L1 diapause stage and during an alternative postembryonic developmental stage called dauer, where it has been shown to affect germ-line stem cell quiescence, dauer maintenance, and survival (21–23). Mutations in the *C. elegans* tumor-suppressor ortholog PTEN/*daf-18* cause similar defects in both dauer and L1 diapause-arrested larvae, although it most probably acts through a genetically independent pathway (15, 24, 25).

AMPK and PTEN Are Both Required for Primordial Germ Cell Quiescence During the L1 Diapause and Post-L1 Diapause Fertility, but Act Through Independent Pathways. When starved AMPK/*aak-1/2* or PTEN/*daf-18* mutant L1 larvae that have been arrested in the L1 diapause are subsequently placed into replete growth conditions, the L1 larvae (which we will refer to hereafter as post-L1 diapause larvae) initiate postembryonic development, although their progression through the various larval stages is slower and more variable than wild-type L1 diapause larvae that were starved for the same duration. Once the surviving post-L1 diapause mutants reach the adult stage they show both somatic and reproductive defects, including a reduced brood size, and many mutant animals die prematurely as adults (Fig. 1*A*, Fig. S1 *A–E*, and Table S1). The survival of the L1 diapause larvae, and the severity and frequency of the somatic growth defects that manifest following recovery of the starved mutant animals are dependent on the duration of the starvation. For example, after 5 d of starvation most animals die, but after 3 d of starvation half the *daf-18* and *aak-1/2* animals were unable to recover, and those that did exhibited a spectrum of somatic and reproductive defects that included vulval defects, sterility, and premature adult lethality. This is not unique to the mutants because we also observe the same types of growth and reproductive abnormalities in wild-type animals that were starved for substantially longer durations (11 d) before transferring them to replete conditions (Fig. 1*B*, Fig. S1 *D* and *E*, and Table S1).

The premature death that occurs in all of the post-L1 diapause animals appears to be independent of the observed morphological somatic defects, because 60% of the post-L1 diapause AMPK/*aak-1/2* or PTEN/*daf-18* mutant adults, and 70% of the wild-type animals that were starved 11 d, die prematurely, 6 d after reaching the L4 stage with no visible somatic defects. Unlike the post-L1 diapause AMPK/*aak-1/2* mutants, *daf-18* adults die earlier than wild-type in a manner that is independent of the L1 diapause (Fig. S1*E*). Therefore, although wild-type animals show a progressive decline in their reproductive fitness during an extended period of starvation (11 d), this same decline occurs in the AMPK/*aak-1/2* and *daf-18* mutants after only a short period of starvation (1–3 d) (Fig. 1 *A* and *B* and Fig. S1 *C* and *D*).

The duration of the starvation at the L1 stage has a cumulative impact on fertility in wild-type, AMPK/*aak-1/2*, and PTEN/*daf-18* animals, whereas the sterility observed in the triple mutant combination (*aak-1; daf-18; aak-2*) is even more severely affected: 71 ± 6% of post-L1 diapause triple mutants became sterile after 1 d in the diapause compared with 4.6 ± 4% and 35 ± 6% for AMPK/*aak-1/2* and PTEN/*daf-18* mutants, respectively. No triple mutants survive after 3 d of starvation at the L1 stage, consistent with AMPK and PTEN acting through independent pathways to ensure survival, robust and timely growth, and somatic and germ-line development following starvation (Fig. S1 *A–D*). After being maintained 3 d in the L1 diapause stage, only 30% of the viable post-L1 diapause *aak-1/2* larvae grew to become fertile adults (Fig. 1*A*). The majority (80%) of these fertile parents display a strikingly reduced brood size, generating fewer than 100 F_1 progeny. We refer to these fertile parents as "reduced" (parents that produce broods with less than 100 F_1 progeny) (Fig. 1*B* and Fig. S1 *C* and *D*). Surprisingly, the remaining 20% of the fertile adults produced an almost normal brood size and seem almost unaffected. We refer to these animals as "normal" (parents that produce more than 100 F_1 progeny).

Because the primordial germ cells (PGCs) undergo inappropriate supernumerary divisions in both AMPK/*aak-1/2*– and PTEN/*daf-18*–starved L1 mutant larvae (Fig. S1*F*) (14, 15), we wondered whether this aberrant proliferation may be responsible for the compromise in fertility and brood size observed in the post-L1 diapause AMPK/*aak-1/2* and PTEN/*daf-18* mutant adults. Using previously described genetic suppressors that block the supernumerary PGC divisions in both PTEN/*daf-18* and AMPK/*aak-1/2* mutants (14, 15, 23), we determined whether they might also restore fertility in the post-L1 diapause mutant adults. Whereas, *age-1* (PI 3-kinase) mutations restore both germ-line quiescence and fertility of post-L1 diapause PTEN/*daf-18* mutants (Fig. S1*G*), *age-1* did not suppress the sterility of AMPK/*aak-1/2* mutants, but alternatively, enhanced sterility in the triple mutants (87± 6% of 3-d post-L1 diapause *age-1; aak-1/2* adults became sterile compared with 66 ± 3% in *aak-1/2* adults) (Fig. S1*G*). Moreover, recent data suggest that blocking the target of rapamycin complex 1 (TOR) pathway by compromising *raga-1* or *ragc-1* can restore PGC quiescence in both AMPK/*aak-1/2*– and PTEN/*daf-18*–starved L1 mutant larvae (14). We observed that *raga-1(lf)* restored the fertility of PTEN/*daf-18* adults that had transited through the L1 diapause, but did not restore the fertility of post-L1 diapause AMPK/*aak-1/2* mutant adults (Fig. S1*G*). This finding suggests that the supernumerary PGC divisions are unlikely to be the sole basis of the sterility seen in post-L1 diapause AMPK/*aak-1/2* mutants, although the supernumerary PGC divisions may contribute to the sterility observed in PTEN/*daf-18* mutants.

Germ-Line and Gonad Defects in Post-L1 Diapause AMPK Mutant Hermaphrodite Adults. To better understand the basis of the AMPK/*aak-1/2* reproductive defects that result from starvation at the L1 stage, we examined the germ lines of adult hermaphrodites (Fig. 1 *C* and *D* and Fig. S2 *A–C*). The gonads of the post-L1 diapause AMPK/*aak-1/2* adults were highly disorganized, often possessing smaller or empty arms. The size of the germ-cell nuclei was variable, whereas the typical germ-cell morphology appeared abnormal in many animals. In wild-type adult hermaphrodites, germ cells exit a mitotically active zone at the distal end of the gonad and enter meiotic prophase I as they move proximally. This region of the gonad called the transition zone is cytologically distinct based on DAPI staining (26). The length of the transition zones seen in post-L1 diapause AMPK/*aak-1/2* mutants was extremely variable and sometimes transition zones were completely absent. The chromosomal morphology typical of cells in the transition zone based on DAPI staining was abnormal, suggesting that the progression from mitosis to meiosis was affected. None of these features were reproducibly

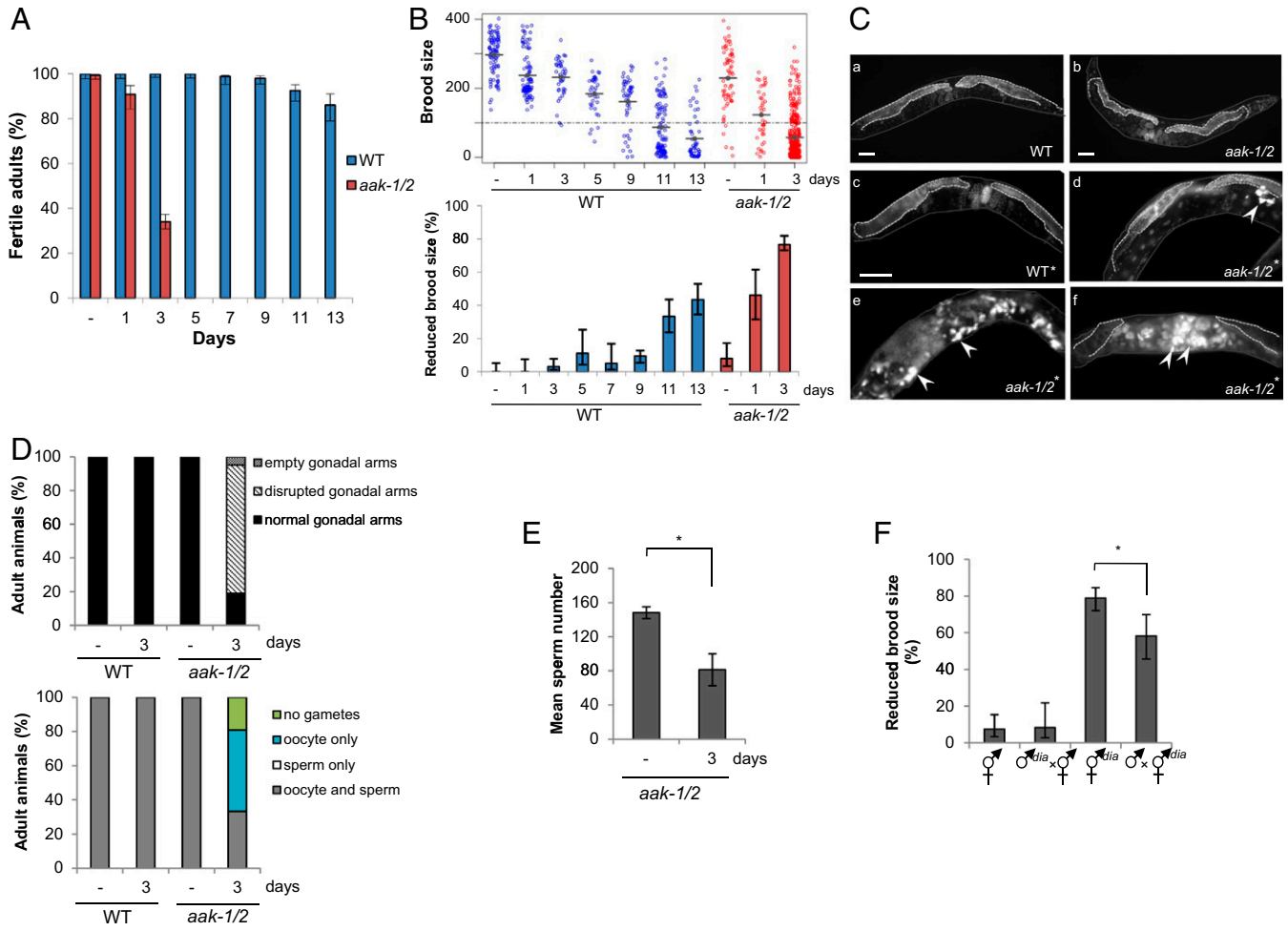

Fig. 1. Loss of AMPK results in sensitivity to acute starvation and subsequent postrecovery reproductive effects. (*A*) AMPK is required to ensure adult reproductive fitness after recovering from L1 starvation. Following varying durations of starvation ("Days" refer to days of starvation here and throughout), post-L1 diapause larvae were singled to replete plates, allowed to grow to adulthood when their fertility was scored. "–" indicates nonstarved animals. Error bars represent the confidence interval at 95% (95% CI); $n \geq 80$ animals were scored per experimental condition. WT, wild type. (*B*) Prolonged starvation in the L1 diapause results in a reduction in the F_1 brood size for post-L1 diapause *aak-1/2* mutant and wild-type animals. Total number of F_1 progeny born from fertile post-L1 diapause parents were scored following varying durations in the L1 diapause [nonstarved (–), 1, 3, 5, 7, 9, 11, or 13 d]. (*Upper*) Brood size distribution for wild-type and *aak-1/2* animals after varying durations of L1 diapause. The average brood size is indicated by horizontal bars for each genotype/condition. The dotted line represents the minimum number of F_1 progeny used to define reduced brood size (fewer than 100 F_1 progeny). (*Lower*) The proportion of individuals with reduced brood size is represented, $n \geq 80$ animals. Error bars: 95% CI. (*C*) L1 starvation disrupts gonad morphology in post-L1 diapause *aak-1/2* adult hermaphrodites. L1 larvae were either allowed to eat immediately (–) or maintained in the L1 diapause for varying durations before they were singled to OP50-seeded plates. Whole-animal DAPI staining was performed on wild-type or *aak-1/2* adult hermaphrodites to visualize the germ cells. The gonads of (*a*) nonstarved wild-type controls or (*b*) *aak-1/2* mutant adult hermaphrodites possess two symmetric, morphologically normal gonadal arms, which are also observed in (*c*) wild-type adults that were previously subjected to a 3-d period in the L1 diapause indicated by the asterisk (*). In contrast, young adult hermaphrodite *aak-1/2* mutants that developed after a 3-d L1 diapause (*) displayed abnormal gonads that lack or have reduced germ cell numbers (*d*–*f*), where the mitosis–meiosis zone is disorganized. In addition, many germ cells undergo endomitotic cell cycles (arrowheads). Dotted lines delineate the gonad boundary, whereas solid gray lines outline the cuticle. (Scale bar, 50 μm.) (*D*) Morphological defects in the gonad are unique to *aak-1/2* mutants that were previously subjected to 3 d in the L1 diapause. Gonadal defects observed in young-adult post-L1 diapause *aak-1/2* mutant adults from *C* were quantified and represented graphically. Oocyte-only animals lack sperm; sperm-only animals lack oocytes. Oocytes and sperm-contain both; $n \geq 20$ animals. (*E*) Sperm numbers are reduced in post-L1 diapause *aak-1/2* mutants. Sperm were counted following DAPI staining in *aak-1/2* mutant adults subjected to none (–), or a 3-d duration in the L1 diapause and subsequent recovery on replete plates until the adult stage; $n \geq 8$ gonad arms. Error bars: SE, $^*P < 0.05$ using Student's *t* test. (*F*) Reproductive defects observed in post-L1 diapause *aak-1/2* mutants are caused predominantly by compromised oocyte integrity with comparatively less contribution from the sperm. Crosses performed with post-L1 diapause hermaphrodites were partially rescued by mating with nonstarved *aak-1/2* mutant males. Reciprocal crosses were performed between post-L1 diapause *aak-1/2* mutant males ($♂^{dia}$), or hermaphrodites ($♀^{dia}$) that were previously maintained 3 d in the L1 diapause. The resulting proportion of animals that exhibited a reduced brood size from successful mating (as judged by 50% frequency of males in the F_1 progeny) was tabulated for comparison. Error bars: 95% CI; $^*P < 0.05$ Fisher's exact test.

observed in AMPK/*aak-1/2* mutant hermaphrodites that were not subjected to the L1 diapause. These germ-line abnormalities likely impinge on gamete integrity because many of the disrupted gonads possessed oocytes, while several of these appear to have undergone endomitotic cycles based on DAPI staining (Emo, 76%, $n = 20$). In addition, most of the post-L1 diapause *aak-1/2* adults exhibit gonads with a strong reduction, or a complete absence of sperm (Fig. 1 *D* and *E* and Fig. S2*C*). This reduced abundance and efficiency of the sperm, compounded with the observed defects in oogenesis after starvation, likely contribute to the observed brood-size defect in post-L1 diapause AMPK/*aak-1/2* mutant hermaphrodite adults.

The observed reproductive defects could result from either somatic or germ-cell deficiencies that arise during or following the L1 diapause. To determine if the defects were a result of the integrity of the oocytes, the sperm, or both, we performed reciprocal crosses and assessed the brood size of the resulting cross progeny (Fig. 1*F*). When we mated AMPK/*aak-1/2* mutant hermaphrodites that had never been starved with post-L1 diapause AMPK/*aak-1/2* males, the resulting brood sizes were normal and we did not observe any significant increase in sterility, suggesting that the abundance or the quality of the sperm in post-L1 diapause AMPK/*aak-1/2* adult hermaphrodites is not a major factor in the observed sterility. However, when the crosses were performed with post-L1 diapause AMPK/*aak-1/2* mutant hermaphrodites and nonstarved male AMPK/*aak-1/2* mutant animals, we noted that the brood-size defects could only be partially restored. A similar partial rescue was observed when nonstarved wild-type males were mated with post-L1 diapause AMPK/*aak-1/2* mutant hermaphrodites. This finding suggests that only some of the reproductive defects (sterility or reduced brood size) of the post-L1 diapause AMPK/*aak-1/2* mutants are derived from the sperm defects described above (Fig. 1*E*). These results collectively suggest that the germ line of the hermaphrodite is particularly sensitive to starvation during this period and that when the PGCs are affected, a defect occurs in the oocytes such that fertility cannot be fully restored through crossing with unaffected males. The memory of the starvation remains molecularly recorded and ultimately affects the integrity of the oocyte.

Transgenerational Reproductive Defects Arise in Post-L1 Diapause AMPK Mutants. This phenomenon is consistent with epigenetic modifications that alter the chromatin, ultimately affecting cellular outcomes over the course of one or more cell divisions or even generations. To test whether a single bout of starvation during the L1 stage could result in transgenerational epigenetic defects in subsequent generations of progeny that never experienced the initial starvation, we carried out three distinct multigenerational analyses based on different hypotheses (Fig. 2 *A* and *B* and Fig. S2*E*). Using the first method (method #1 in Fig. 2*A*), we postulated that post-L1 diapause parents that have a reduced brood size (fewer than 100 F_1 progeny) might have an increased chance of transmitting these epigenetic changes transgenerationally compared with post-L1 diapause parents that exhibit a normal brood size (more than 100 F_1 progeny). To test this hypothesis, we followed animals through multiple generations by continuously selecting L4 larvae born from parents that produced a reduced brood size at each generation. The larvae were then allowed to develop, produce progeny, and their individual brood sizes were evaluated and recorded for each generation (F_x).

Alternatively, it is possible that all of the post-L1 diapause AMPK/*aak-1/2* mutant adults are affected by the stress of starvation during the L1 diapause, regardless of whether the parents exhibit reproductive defects initially. We noted that some (20%) of the fertile post-L1 diapause AMPK/*aak-1/2* mutant adults had normal broods, but their progeny fell into two categories: those that generated F_2 broods that were reduced in size and those that produced normal F_2 broods. By systematically selecting L4 larvae from each generation (*Materials and Methods*), we then tracked individuals from the reduced and normal categories and constructed multigenerational lineages that were based on whether they were initially selected from parents that had a reduced brood size (method #2), or whether they were generated from parents that had a normal brood size (method #3) (Fig. 2*A*; additional information in *Materials and Methods*).

All of the animals that were tracked through successive generations using method #3 generated progeny with comparatively less impact on brood size. The minor effects that did manifest in the early generations were resolved rapidly, but in later generations (F_5–F_9) reproductive defects did appear, despite our continuous selection of normal parents. This finding suggests that even though most of these post-L1 diapause parents were either phenotypically unaffected by the diapause, or that any initially occurring defects may have been resolved in the first generation, some heritable record of the initial event persisted and was manifested, albeit at low penetrance, up to nine generations after the initial event.

Alternatively, when we continuously selected post-L1 diapause AMPK/*aak-1/2* descendants from parents that had reduced brood sizes using methods #1 or #2, we consistently observed transgenerational brood-size defects (Fig. 2*B*). Curiously, this transmissible brood-size defect occurred despite the fact that these successive generations of progeny had never been starved or, in some cases, whether their parents appeared reproductively compromised or not (method #2). Therefore, regardless of whether the initially affected *aak-1/2* post-L1 diapause animals had a normal or reduced brood size, both types of parents are very likely to transmit this reduction in brood size to their descendants (Fig. 2*B*). This progressive transgenerational reduction in reproductive fitness is never observed in nonstarved AMPK/*aak-1/2* mutant animals, in 3-d post-L1 diapause PTEN/*daf-18* animals, or in 3-d or 11-d starved wild-type animals (Fig. S2*E*).

There is a substantial degree of variability in the brood-size defects between these parental lineages that may represent a graded threshold effect in response to the epigenetic stimulus (starvation). However, this variability seems typical of an epigenetic response where not all of the germ cells are affected by starvation in an identical way, potentially because of individual differences in availability to nutrient/energy levels within the PGCs. Nevertheless, most AMPK/*aak-1/2* mutant animals that survive the acute stress associated with starvation in the L1 diapause will produce progeny that will eventually be adversely affected by the initial challenge, even though the affected animals in subsequent generations may never have experienced the stress directly.

To determine the extent to which each individual animal was affected we quantified the brood size of 10 individual fertile post-L1 diapause AMPK/*aak-1/2* mutant parents and that of their progeny over several generations (Fig. 2*C* and Fig. S2*F*). Nine of these lineages (P_0-I to P_0-IX) were analyzed using method #1 and one lineage (P_0-X) was analyzed using method #2. In each of the parental lineages analyzed by method #1 (P_0-I to P_0-IX) we observed a similar progressive reduction in brood size that varied in expressivity and in the delay that preceded either the correction of the reproductive defects or the eventual terminal arrest of the lineage. This progressive brood size reduction is similar to the progressive mortal germ-line phenotypes (Mrt) that remain fertile indefinitely at permissive temperature but become progressively sterile after growth for multiple generations at higher temperatures (27). These phenotypes are typically caused by mutations in genes involved in genome integrity or epigenetic regulation (27–30). Consistent with this, in four independent lineages (see † in Fig. 2*C*), we noted that animals became very sickly in late generations, segregating a high percentage of male progeny (Him) and other spontaneous mutant phenotypes [Dumpy (Dpy), Roller (Rol), Long (Lon), Small (Sma)]: in the F_6 plates of P_0-VIII and the F_8 plates of lineage P_0-IX, 9% and 14% of the animals segregate a strong Him phenotype in next generation; 6% and 14% segregate a Lon phenotype; whereas 3% and 5% give rise to a Dpy phenotype, respectively, before laying a large number of dead eggs. In the animals present on the F_3 plates of the P_0-VI and P_0-VII lineages, up to 33% of the progeny segregate a Sma phenotype, 4–8% give rise to a Him phenotype, and 80–92% of the remaining viable progeny eventually arrested between the L1 and L3 stages.

We were able to correct the brood-size defects of post-L1 diapause AMPK/*aak-1/2* mutant descendants from later generations in P_0-VIII and P_0-IX by crossing them with nonstarved wild-type (N2) males (Fig. 2*D*). However, although AMPK/*aak-1/2* mutant males could partially suppress the brood-size defect in the first generation of affected animals born from post-L1 diapause

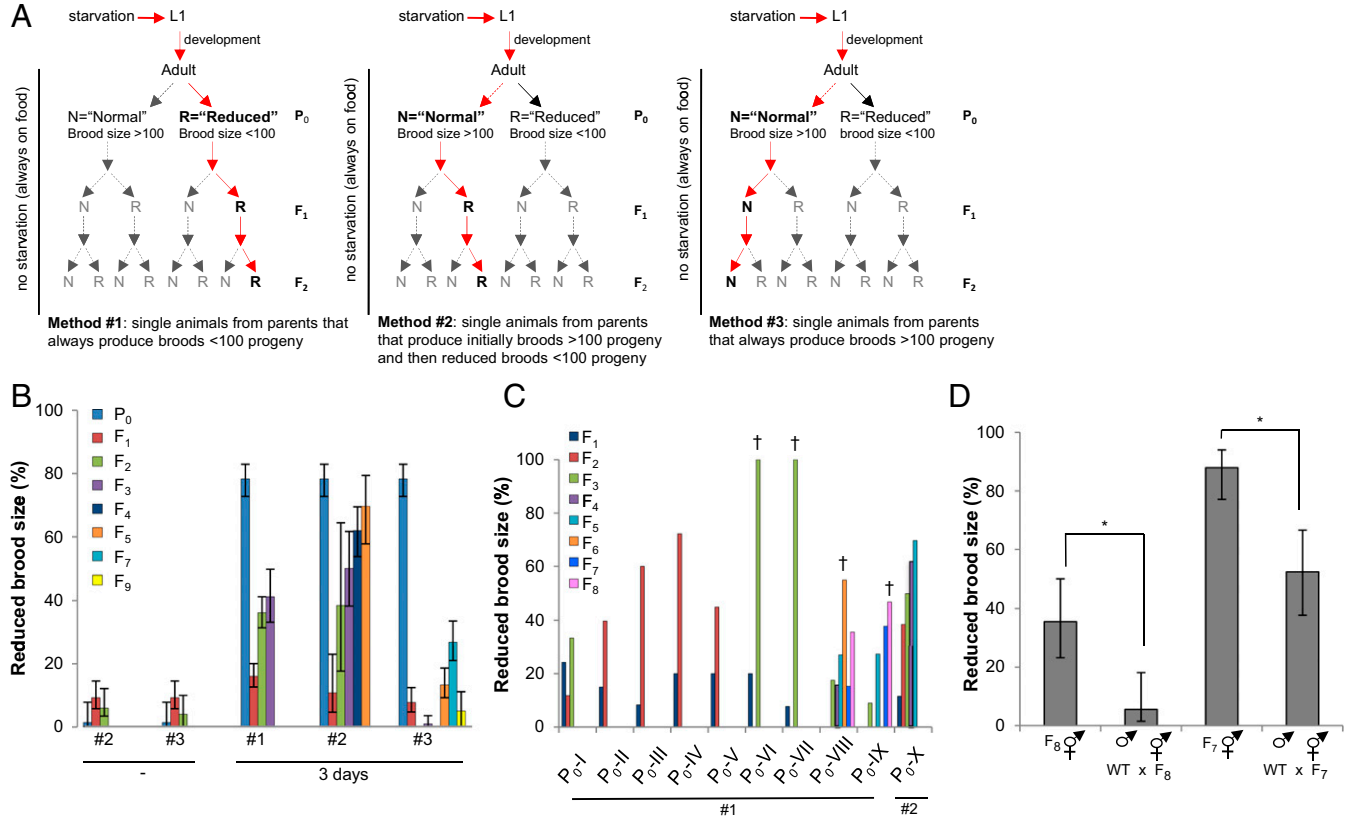

Fig. 2. Post-L1 diapause AMPK mutants display variable transgenerational reproductive defects. (*A*) Schematic representation of the three selection methods used for our transgenerational analyses. Method #1: transgenerational analysis performed using fertile parents (P_0) that have a reduced brood size of less than 100 F_1 progeny. Methods #2 and #3: Multigenerational analyses were carried out first using parents that produce a normal brood size of more than 100 F_1 progeny (see *Materials and Methods* for additional details). Red arrows indicate the selection at each successive generation. (*B*) Loss of AMPK causes transgenerational reproductive defects following a short duration in the L1 diapause. The percentage of post-L1 diapause animals that generated descendants that bore smaller broods (≤100 progeny) after either no starvation (–), or 3 d in the diapause. For multigenerational analyses, the F_1 progeny generated from reduced parents using method #1, or parents with a normal brood size using methods #2 and #3 were analyzed over multiple generations (see Fig. S2*E* for additional information). Error bars: 95% CI. (*C*) *aak-1/2* mutants exhibit a progressive transgenerational reduction in brood size. Phenotypic variability in brood size is inherent in subsequent generations analyzed from fertile post-L1 diapause *aak-1/2* parents. Brood sizes were analyzed over multiple generations in the descendants of 10 independent post-L1 diapause *aak-1/2* mutant parents. Brood-size defects were monitored in lineages P_0-I to P_0-IX using section method #1, whereas for comparison, the analysis in P_0-X was performed using method #2. Descendants that showed spontaneous mutant phenotypes that did not subsequently breed true (Him, Lon, Dpy or Sma) are shown by a dagger (†). (*D*) The multigenerational reproductive defects that occur in post-L1 diapause *aak-1/2* mutant animals arise because of adverse epigenetic changes that occur as a result of the absence of AMPK. *aak-1/2* transgenerational brood-size defects are only partially rescued by the introduction of a wild-type copy of AMPK. Well-fed wild-type males (nonstarved) were crossed with *aak-1/2* hermaphrodites that were selected from two independently maintained transgenerationally compromised lineages (P_0-VIII and P_0-IX) that produced smaller broods (≤100 progeny) after seven or eight generations following the initial diapause (F_7 and F_8, respectively). The brood sizes of cross progeny were determined and represented as the percentage of the total population of animals born from the same successful cross. Error bars: 95% CI; *$P < 0.05$ Fisher's exact test.

AMPK/*aak-1/2* mutant parents (Fig. 1*F*), crossing nonstarved AMPK mutant males with these later generation (F_4 or F_5) AMPK/*aak-1/2* mutants did not suppress the brood-size defects in the resulting cross progeny (Fig. S2*G*; compare with Fig. 1*F*). These observations are consistent with an epigenetic phenomenon that affects germ-line integrity in the post-L1 diapause AMPK/*aak-1/2* mutants that becomes progressively worse with each successive generation. Furthermore, although this transgenerational defect can be corrected by wild-type gene activity, the inability of nonstarved AMPK/*aak-1/2* mutant males to rescue the transgenerational brood size defects of the post-L1 diapause AMPK/*aak-1/2* mutant hermaphrodites suggests that AMPK activity may also be required to resolve these epigenetic modifications once they are established.

The possibility that the stress of an initial starvation event might confer increased resistance to a subsequent bout of starvation as an evolutionary trade-off was excluded because F_1 animals obtained from fertile post-L1 diapause AMPK/*aak-1/2* mutant adults exhibited similar L1 survival rates to animals that had never been previously starved. The F_1 animals that arose from post-L1 diapause AMPK/*aak-1/2* mutant parents with reduced broods did, however, die earlier than nonstarved mutant controls (Fig. S2*H*; see asterisks, F_1*).

Our data therefore indicate that both PTEN/*daf-18* and AMPK are required for survival and adult fertility in response to acute starvation during the early L1 stage. Whereas PTEN/*daf-18* is required acutely in a pathway that is independent of AMPK, its effects are resolved in the first generation following the starvation. In AMPK/*aak-1/2* mutants however, the reproductive consequences that manifest in the post-L1 diapause animals persist throughout multiple generations after the initial event resulting in the progressive extinction of the affected lineages.

H3K4me3 Levels Are Abnormally High in the PGCs of Post-L1 Diapause AMPK Mutants. Changes in chromatin modification have been linked to epigenetic transmission in several contexts (31). To determine whether chromatin marks are affected in AMPK/*aak-1/2* mutants following starvation in the L1 stage and whether

these marks persist in the subsequent affected generations, we examined the levels of various histone modifications in the PGCs of post-L1 diapause wild-type and mutant L1 larvae. Consistent with recent reports, the levels of phosphorylated H2B were reduced in starved AMPK/*aak-1/2* mutants (18), whereas the levels of H3K4me3 were increased in the PGCs of starved AMPK/*aak-1/2* mutants (Fig. 3 *A* and *B* and Fig. S3 *A* and *B*). Emergent L1 larvae in the subsequent F_2, F_4, and F_6 generation derived from post-L1 diapause AMPK/*aak-1/2* mutants also possess increased levels of H3K4me3 (Fig. 3*B*).

In many organisms H3K4me3 is catalyzed by a COMPASS-like complex, the catalytic core of which includes a histone methyltransferase of the Su(var)3-9/enhancer of zeste/trithorax domain protein (SET1)/mixed lineage leukaemia (MLL) family (32–34). The SET1/MLL orthologs in *C. elegans* are SET-2 and SET-16 (35, 36) and they exist in a large multisubunit assembly (34, 37). Mutations in the COMPASS-like complex exhibit somatic phenotypes that include disruptions in vulva development by attenuating LET-60/RAS signaling, and a reduced brood size (35, 36). We noted that increases in H3K4me3 levels, such as those seen in mutants that harbor mutations in *rbr-2*: a member of a family of the histone demethylases involved in removing methyl groups from H3K4, lead to somatic defects, reduced brood size, and premature adult death (38). These phenotypes mirror those we observed in our post-L1 diapause AMPK/*aak-1/2* mutants, suggesting that the misregulation of H3K4me3 levels may be linked to the observed defects (Fig. S3 *C* and *D*).

Consistent with the increases in H3K4me3 observed in the PGCs of the starved AMPK/*aak-1/2* mutants, we also observed a global increase in H3K4me3 in later-stage (L4) post-L1 diapause animals, suggesting that early modifications that occur in the PGCs are not reduced to a baseline threshold more typical of wild-type upon the onset of postembryonic development, but rather remain abnormally elevated (Fig. 3*C*). The increased H3K4me3 levels were not unique to the germ cells, but also occurred in the soma. Larvae that were subjected to *gon-1(RNAi)*, which compromises germ-cell proliferation without affecting nongonadal cells (39), retained significantly high levels of H3K4me3 despite a reduction in the number of germ cells at this stage (Fig. 3*C*).

If the abnormal accumulation of H3K4me3 in the PGCs during starvation is responsible for the fertility defects and reduced brood size in the post-L1 diapause AMPK/*aak-1/2* mutants, then reducing their levels should suppress the reproductive defects seen in both post-L1 diapause AMPK/*aak-1/2* mutants and their descendants.

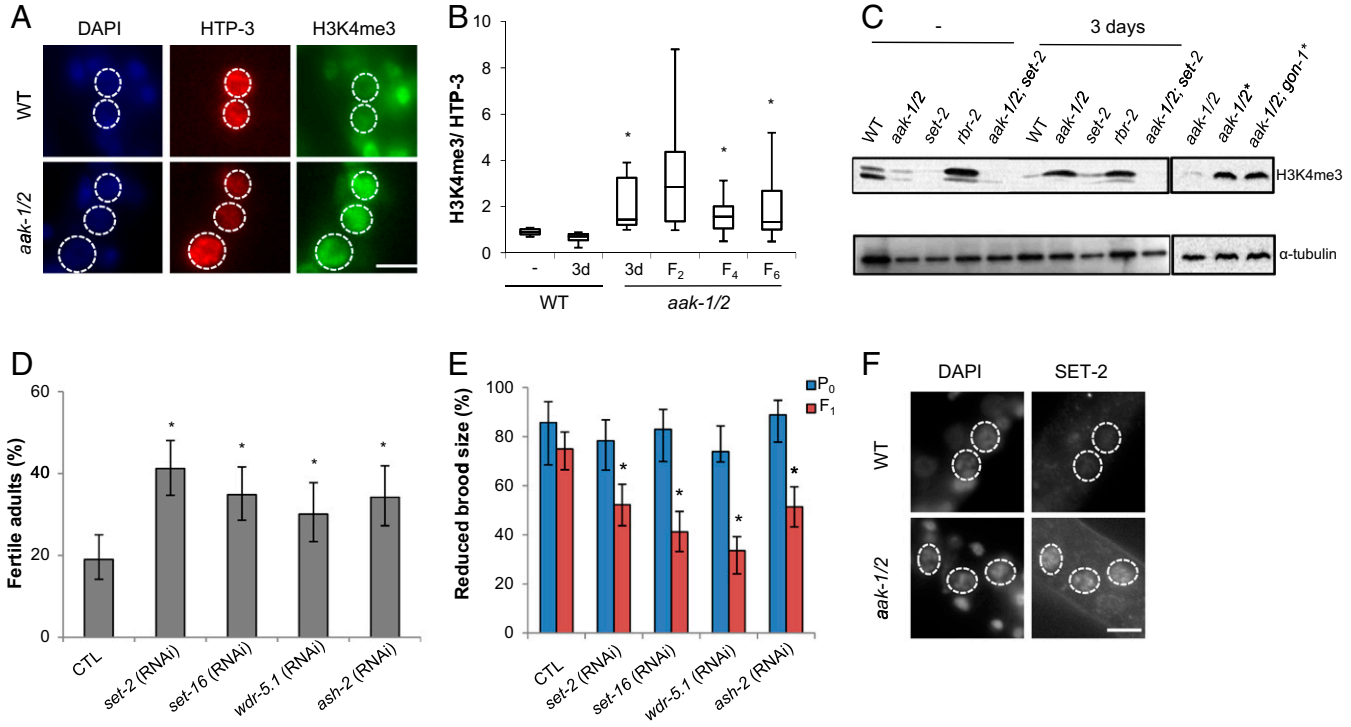

Fig. 3. H3K4me3 levels accumulate and persist over multiple generations in the primordial germ cells of post-L1 diapause AMPK mutants. (*A*) H3K4me3 is increased in the PGCs of post-L1 diapause *aak-1/2* mutants. Emergent wild-type and *aak-1/2* L1 larvae were starved 3 d before fixation and immunostaining with H3K4me3 (green) and the PGC-specific marker HTP-3 (red) antibodies. Dashed lines mark the PGCs; note that the PGCs undergo supernumerary divisions in the *aak-1/2* mutants, as previously described. (Scale bar, 5 μm.) (*B*) H3K4me3 is elevated through multiple generations in the progeny of post-L1 diapause *aak-1/2* L1 mutants. Quantification of H3K4me3 levels normalized to HTP-3 signal following immunostaining in the PGCs of nonstarved (–), 3 d starved (3d), and in subsequent generations (F_2, F_4, and F_6) of post-L1 diapause animals. $n \geq 8$ different animals from which all PGCs were used for the quantification. $^*P < 0.05$ (one-tailed *t* test). (*C*) The elevated H3K4me3 levels that arise in PGCs of starved *aak-1/2* mutants occur in both the germ line and in the soma. The modification persists into later stages of development and is dependent on the SET-2 histone methyltransferase. Wild-type, *aak-1/2, set-2*, and *rbr-2* post-L1 diapause larvae that spent 3 d in the diapause, or not (–), were recovered to replete plates and collected at the mid-L4 stage for immunoblot analysis using an anti-H3K4me3 antibody. Forty percent more post-L1 diapause (*) *aak-1/2; gon-1(RNAi)* animals were required to match the levels of the α-tubulin loading control. (*D* and *E*) Increased COMPASS complex activity contributes to sterility and brood-size defects in post-L1 diapause *aak-1/2* mutants. Compromise of COMPASS complex components by dsRNA soaking during the period of starvation improves fertility (*D*), and the frequency of animals that exhibit brood size defects (*E*) in the F_1 descendants of post-L1 diapause *aak-1/2* mutants. Control animals (CTL) are *aak-1/2* L1 larvae maintained 3 d in M9 buffer containing GFP dsRNA. Error bars: 95% CI; $^*P < 0.05$ Fisher's exact test, (*D*) $n \geq 150$, from three independent experiments. (*E*) For P_0 analysis $n \geq 40$, and for the F_1 analysis $n \geq 120$ from three independent experiments. (*F*) PGCs of starved *aak-1/2* mutants have abnormally high levels of SET-2. Immunostaining of SET-2 after 3 d of starvation in wild-type and *aak-1/2* mutant L1 larvae. Animals were counterstained with DAPI. Dotted white lines delineate the boundaries of the PGC nuclei. (Scale bar, 5 μm.)

Consistent with a misregulation of the COMPASS complex in the observed starvation-dependent sterility, soaking emergent L1 larvae in buffer containing double-stranded RNA (dsRNA) corresponding to any one of the COMPASS components *set-2*, *set-16*, *ash-2*, or *wdr-5.1* for a 3-d period during the L1 diapause was sufficient to partially suppress both the sterility of the post-L1 diapause AMPK/*aak-1/2* mutant larvae and the reduced brood size observed in the F_1 generation. Conversely, the same treatment did not affect the fertility or the brood size of wild-type animals (Fig. 3 *D* and *E* and Fig. S3 *E* and *F*). Furthermore, the compromise of *set-2* was sufficient to dramatically reduce the global H3K4me3 levels in the post-L1 diapause AMPK/*aak-1/2* mutant larvae (Fig. 3*C*).

Curiously, most of the vulval defects of starved AMPK/*aak-1/2* mutants were not suppressed when L1 larvae were treated with dsRNA corresponding to *set-2* or *set-16*, suggesting that other H3K4me3-independent pathways may be disrupted in the post-L1 diapause AMPK/*aak-1/2* larvae (Table S1). Although the suppression that we achieve by removing components of the COMPASS complex is partial, this may reflect our inability to remove all of the target protein through RNAi. On the other hand we cannot rule out that other factors that may contribute to the observed defect that are not downstream of inappropriate histone methyltransferase activity.

AMPK has been demonstrated to drive adaptive metabolic adjustment to energy stress by phosphorylating key cellular proteins, ultimately blocking anabolic pathways while simultaneously enhancing pathways involved in energy production or conservation. Bioinformatic analysis revealed that all of the members of the COMPASS-like complex components, except DPY-30, possess amino acid signatures that match the optimal AMPK substrate motif (Fig. S3*G*) (40). This finding represents a significant enrichment of these sites within this complex, suggesting that AMPK may regulate the activity of the complex during environmental challenges. Despite our efforts we could never acquire the quantity of PGCs to confirm this possibility biochemically. Moreover, the transient nature of the phosphorylation makes it unlikely that it would be present in the germ cells of later-stage post-L1 diapause larvae/adults.

To determine if AMPK might affect COMPASS function within the starved PGCs by altering its abundance, we examined the levels of SET-2 in the PGCs in starved L1 mutant larvae to assess if its stability or its localization might be affected in an AMPK-dependent manner. We noted that SET-2 levels were consistently higher in the PGCs of starved L1 larvae that lack AMPK compared with starved wild-type PGCs, indicating that AMPK compromise may affect its accumulation in the PGC nuclei and consequently increase the levels of H3K4me3 in the germ cells of the starved AMPK/*aak-1/2* mutants (Fig. 3*F*).

***par-5* Acts with AMPK to Ensure Germ-Line Integrity in a Manner That Is Independent of *set-2*.** AMPK commonly regulates its targets by generating phosphorylated motifs that are recognized and bound by 14-3-3 proteins (40, 41). The 14-3-3 proteins are involved in many diverse biological processes and affect their targets by multiple mechanisms. If AMPK-dependent phosphorylation of SET-2 or other components of the COMPASS complex results in recognition of the phosphotargets by 14-3-3 proteins to mediate its effects downstream of nutrient/energy stress, then loss of the *C. elegans* 14-3-3 proteins should recapitulate the phenotypes typical of AMPK compromise in post-L1 diapause animals. In *C. elegans* there are two predicted 14-3-3 proteins: *ftt-2* and *par-5*, but only *par-5* is expressed in the germ line (42, 43). Because *par-5* is an essential gene we performed RNA soaking experiments with dsRNA corresponding to *par-5* during the L1 diapause. Post-L1 diapause wild-type larvae subjected to *par-5(RNAi)* exhibited pronounced sterility compared with control animals, and this effect could not be compounded by removing AMPK function, indicating that the two gene products likely work in a linear genetic pathway to affect adult fertility following starvation (Fig. 4*A*). In addition, the fertility of both the F_1 and F_2 generation descendants that were derived from post-L1 diapause wild-type animals subjected to *par-5(RNAi)* were also affected (Fig. 4*B*). Therefore, the effect of the single *par-5(RNAi)* treatment during the L1 diapause spanned at least two generations. The similarity of the *par-5(RNAi)* phenotype to that of the observed AMPK post-L1 diapause mutants, and the observation that the compound mutations are not additive, is consistent with a role for PAR-5 in mediating the ability of AMPK to protect germ-line integrity during periods of starvation.

If AMPK triggers a PAR-5–mediated response to modulate COMPASS activity through its regulation of SET-2 accumulation in the PGC nuclei, then the complete removal of *set-2* from *par-5*–compromised starved animals should suppress any inappropriate H3K4me3 caused by accumulation of SET-2, and ultimately improve the fertility of post-L1 diapause mutant animals. This is, however, not what we observe in the *set-2; par-5(RNAi)* animals, which exhibit a substantially greater frequency of sterility than either single mutant alone following a 3-d period in the L1 diapause (Fig. 4*C*). These data strongly suggest that *par-5* and *set-2* do not function in a linear genetic pathway, but rather the two gene products work independently of one another, whereby both are dependent on AMPK function. Therefore, in response to acute starvation AMPK regulates at least two different pathways that ultimately affect germ-line integrity, and its absence during the L1 diapause has reproductive consequences that can persist through multiple generations. One branch appears to be mediated by the 14-3-3 protein PAR-5, which likely targets effectors that have yet to be elucidated, whereas a second branch impinges on the chromatin-writing COMPASS complex.

We propose that during starvation AMPK blocks the catalysis of H3K4me3 in the two PGCs through its ability to modify one or more of the COMPASS components (SET-16, SET-2, ASH-2, WDR-5.1). In post-L1 diapause AMPK mutant larvae, the consequent increase of H3K4me3 in the PGCs is associated with a progressive transgenerational deterioration in reproductive fitness.

Misregulated Transcriptional Elongation Contributes to the Sterility of Post-L1 Diapause AMPK Mutants. The early stages of animal development are critical for reading and writing new epigenetic

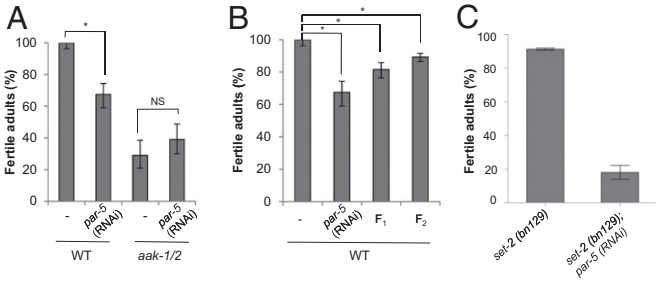

Fig. 4. The 14-3-3 protein PAR-5 acts with AMPK, but in parallel to the COMPASS complex, to protect germ-line integrity. (*A*) Wild-type and *aak-1/2* mutant L1 larvae were hatched then maintained in M9 containing *par-5* dsRNA for 3 d before recovery on food. Adult fertility was assessed in control nonstarved and *par-5(RNAi)* animals; $n = 100$. Error bars: 95% CI; $*P < 0.05$ Fisher's exact test; NS, nonsignificant. (*B*) The 14-3-3 compromise results in transgenerational reproductive defects. Starved wild-type L1 larvae subjected to *par-5(RNAi)* soaking or not (–) for 3 d were recovered and allowed to produce F_1 and F_2 progeny. The number of fertile adult animals in each generation was evaluated and represented; $n \geq 250$. Errors bars: 95% CI; $*P < 0.05$ Fisher's exact test. (*C*) *set-2* and *par-5* function in parallel pathways to establish or maintain post-L1 diapause germ-line integrity. Genetic analysis was performed by soaking *set-2(bn129)* mutants in dsRNA corresponding to *par-5*, as described above.

information directed by maternal and/or paternal instruction (44, 45). How this information is transduced to affect gene expression in response to a given stimulus is currently speculative at best. During starvation in *C. elegans* L1 larvae, a specific gene-expression program is initiated (46) and during this period RNA polymerase II is observed in different configurations: in the "docked" state RNA polymerase II is on proximal promoters waiting for cues to initiate, whereas in the "paused" configuration the complex remains in a postinitiated state, presumably on hold until a physiological or developmental contingency is satisfied (47, 48).

This developmental pausing is not exceptional and in many organisms RNA polymerase II is maintained in a postinitiated state where subsequent elongation is regulated by factors that associate with the phosphorylated carboxyl-terminal domain of the RNA polymerase II large subunit (49). In the PGCs the role of carboxyl-terminal domain phosphorylation appears to be less critical than in the somatic cells, whereas orthologs of some of the critical known elongation regulators are absent from the *C. elegans* genome, suggesting that regulation of elongation is independent of many of the mechanisms described in somatic cells (50). The COMPASS complex may nevertheless contribute to this transcriptional switch in the PGCs in a manner that is independent of the soma-specific controls and therefore must be neutralized in the PGCs during starvation.

To determine if the defects observed in AMPK-compromised starved animals are related to the link between increased H3K4me3 and aberrant resumption of transcriptional elongation, we blocked any elongating complexes during the period of starvation using 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB), a potent, highly specific, elongation inhibitor (51, 52) and quantified the effects on fertility in the treated animals and on the brood-size defects in subsequent generations. Treatment of starved AMPK/*aak-1/2* mutant L1 stage larvae with DRB partially suppressed the sterility observed in post-L1 diapause AMPK/*aak-1/2* mutant adult hermaphrodites by 49% ($n = 100$) (Fig. 5*A*), without affecting the fertility of treated post-L1 diapause wild-type. Moreover, consistent with AMPK and PTEN affecting two independent pathways that control PGC integrity, DRB treatment had no effect on the sterility observed in post-L1 diapause PTEN/*daf-18* mutants (Fig. S1*H*).

The brood-size defects typical of the F_1 generation of post-L1 diapause AMPK mutants were also reduced, but the effect in the F_2 generation is also accompanied by a significant number of affected F_1 progeny animals that resolve their brood-size defects, making it difficult to conclude whether the transgenerational defects are indeed dependent on aberrant elongation in the starved PGCs of AMPK mutants (Fig. 5*B*). Nevertheless, by specifically targeting the aberrant elongating complexes we were able to correct many of the reproductive defects typical of the post-L1 diapause AMPK/*aak-1/2* mutants.

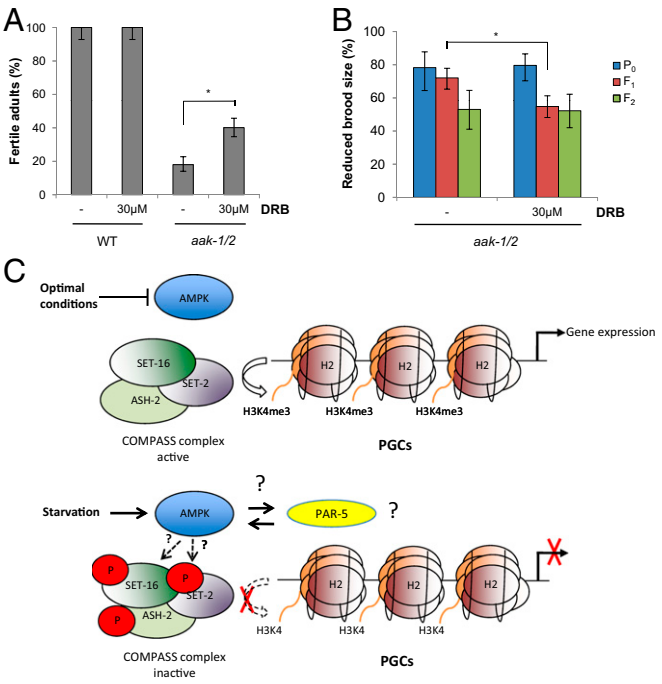

Fig. 5. Inappropriate transcriptional elongation during periods of starvation contributes to the reproductive defects seen in AMPK mutants. (*A*) The elongation inhibitor DRB can partially suppress the sterility observed in post-L1 diapause AMPK mutant hermaphrodites. Emergent wild-type and *aak-1/2* larvae were maintained in M9 buffer containing 30 μM DRB or not (–) for 3 d before recovery on replete plates. The proportion of fertile adult animals is represented, $n \geq 150$. Error bars: 95% CI; $^*P < 0.05$ Fisher's exact test. (*B*) Transgenerational brood-size defects are resolved more efficiently in the progeny of post-L1 diapause AMPK mutant larvae that were treated with DRB during the period of starvation. The frequency of animals with reduced brood size in *aak-1/2* mutants and in the subsequent generation (F_1 and F_2) is represented, $n \geq 50$. Errors bars: 95% CI; $^*P < 0.05$ by Fisher's exact test. (*C*) AMPK affects quiescence and integrity of the PGCs during acute starvation. During replete conditions, AMPK is inactive permitting the COMPASS complex to actively methylate H3K4, thereby enhancing transcriptional elongation. During periods of acute starvation, AMPK becomes activated and targets components of the COMPASS complex, effectively blocking the histone methyltransferase and ultimately inhibiting transcriptional elongation, and hence germ-line gene expression. In addition, AMPK functions with 14-3-3/PAR-5 to affect germ-line integrity through a SET-2–independent pathway.

Discussion

By regulating its various protein targets, AMPK ensures that the PGCs respond efficiently to starvation during the L1 diapause by eliciting both cell cycle and developmental/reproductive quiescence. In its absence, the nutrient/energy contingency is relaxed, allowing a critical chromatin writer, such as the COMPASS complex, to aberrantly activate a postembryonic transcriptional program in the PGCs during a period when all cells should remain quiescent (Fig. 5*C*).

It is unclear why the chromatin modifications that arise in the post-L1 diapause AMPK mutants cannot be resolved in subsequent generations, but instead accumulate to progressively extinguish the germ line. The heritable effects of *set-2* mutations on lifespan are largely resolved in a limited number of generations (53). In contrast, the transgenerational defects of post-L1 diapause AMPK mutant descendants often worsen and cannot be corrected by crossing with mutants that were never starved. In addition, the sterility observed in wild-type animals that were subjected to an 11-d L1 diapause is completely corrected in the next generation. However, if AMPK is reduced in these animals as they grow to adulthood, the fertility of the next generation of animals is visibly affected, albeit not significantly (Fig. S2*I*). This finding suggests that similar epigenetic modifications can occur even in wild-type animals after severe starvation, and AMPK may be important for the resolution of these modifications in the subsequent generation. In the absence of AMPK function, these chromatin modifications become more abundant and can be transmitted to subsequent generations. The germ-line defects we describe herein resemble those seen in *spr-5* mutants that have compromised demethylase function, where the germ line becomes mortalized over several generations (28, 54). Understanding the mechanism through which AMPK functions with PAR-5 to ensure PGC integrity may provide some insight regarding the potential targets that exacerbate the progressive germ-line extinction that occurs in the descendants of post-L1 diapause AMPK mutants.

We have shown that during periods of acute starvation in the L1 stage, the absence of AMPK results in the inappropriate

writing of epigenetic marks that compromise germ-line integrity because of this change in gene expression. The L1 diapause, and potentially other diapause states, such as the dauer, may be critical developmental buffer zones, allowing the animal to couple appropriate stasis of the chromatin landscape to environmental conditions. Our data suggest that AMPK is a pivotal molecular link to couple the chromatin environment and consequent adaptive changes in gene expression, with the metabolic status of the animal to ensure proper regulation of gene expression during this extended developmental hiatus. Recent data have demonstrated that starvation can induce the expression of specific RNAs that may provide some selective advantage in subsequent generations (10). It will be particularly exciting to determine if the expression of this suite of RNAs may be coupled to AMPK function to trigger a heritable adaptive change in the germ line during periods of severe energy stress.

Our data delineate how an AMPK-mediated adaptation during acute starvation includes the buffering of epigenetic writers and is critical to preserve germ-line integrity. This is presumably achieved by adjusting energy resources accordingly in part by limiting enzyme activities that could otherwise compromise the fitness of the animal. How general this AMPK function may be is currently unknown, nor can we speculate on the limits of its buffering capacity. At present, there are no polymorphisms that have been characterized that affect AMPK function and that are associated with the affected Hongerwinter victims or their children. However, our findings suggest that failure at any level of this complex cascade downstream of AMPK activation could have far-reaching implications. Variations in an AMPK-mediated response could potentially account for the frequency and spectrum of observed genetic and epigenetic anomalies that manifest in these individuals and in other survivors of famine.

Materials and Methods

C. elegans Strains and Culture. *C. elegans* were cultured at 20 °C on OP50, as previously described (55) unless otherwise indicated. Strains used in this study include: Bristol N2 wild type, *aak-1(tm1944)I*, *aak-2(ok524)X*, *daf-18 (ok480)IV*, *raga-1(ok386)II*, *age-1(hx546)II*, *set-2(ok952)III*, *set-2(n4589)III*, *set-2(bn129)III*, *rbr-2(tm1231)IV*.

Postrecovery Defects Following the L1 Diapause. The L1 starvation assay was adapted from previously described protocols (56, 57). Briefly, animals were fed with OP50 for at least five generations before any analysis. Gravid hermaphrodites were treated with alkaline hypochlorite and the resulting eggs were hatched and cultured in sterile M9 medium in 15-mL tubes with rotation at 20 °C for the duration of the diapause. After 24 h, the density of newly hatched L1 larvae was adjusted to 6–10 L1 larvae per microliter. To determine viability, 10-μL aliquots were distributed on 6-cm nematode growth medium (NGM) plates seeded with OP50 every 48 h and the number of L1 larvae was counted (initial). The following day, the number of moving animals was recorded as viable, and the survival rate was calculated as viable animals per initial number seeded. This experiment was repeated twice per time point. A minimum of three independent experiments was performed per analysis; the average survival at each time point was determined and used to generate the survival curve where error bars indicate SD.

Analysis of Fertility, Brood Size, and Viability. Newly hatched larvae were maintained in the absence of food and every 48 h groups of L1 larvae were transferred to OP50-seeded NGM plates and maintained at 20 °C until the L2 stage, when they were singled onto seeded NGM plates and allowed to grow to adulthood. Larval growth and other postembryonic developmental phenotypes (Bag, Burst, Evl, Muv) were monitored. Fertility and other phenotypic abnormalities (presence of dead embryos, males, long adults, premature death) were assessed up until 1-wk post-L4 stage. All experiments were performed at least three times; 200–800 recovered animals were analyzed per genotype per time point were monitored.

Brood-size analysis was performed by counting progeny born from fertile parents of varying genotype. Fertile post-L1 diapause AMPK mutant adults were either parents that generated a reduced brood size (fewer than 100 F_1 progeny), or those parents that produced a normal brood size (more than 100 F_1 progeny). To quantify brood size, post-L1 diapause hermaphrodites were allowed to develop to the L4, after which they were singled to plates and were allowed to reproduce and were transferred daily throughout their entire reproductive period (3 d). The number of F_1 offspring that were present on each plate that reached the L4/young adult stage was thereafter counted, allowing us to infer whether the parent had a normal or reduced brood size. Fifty to 150 animals were analyzed per genotype per time point from three independent experiments.

The viability of post-L1 diapause hermaphrodites was scored every 48 h following the L4 stage after varying durations in the diapause (0, 1, 3, 11 d); 100–300 animals were evaluated from three independent experiments.

Progressive Brood-Size Reduction in Individual Parental Lineages. Ten independent post-L1 diapause *aak-1/2* mutant lineages were analyzed independently. For each lineage, the entire population of F_1 progeny from post-L1 diapause *aak-1/2* fertile parents (P_0) was singled onto fresh plates with food. Brood-size defects were quantified from individual fertile parents by counting progeny. Plates with animals that exhibited a reduced brood size were kept to continue the analysis and 100–200 individuals per parental lineage were singled every generation onto replete plates. The transgenerational progeny obtained from individual starting lineages were maintained separately for the duration of the analysis. In the final generations of several lines, the animals became very sick and the proportions of plates that possessed animals that showed a Him, Lon, Dpy, or Sma were assessed.

Transgenerational Rescue. To determine the role of sperm in the post-L1 diapause reproductive defects 3-d post-L1 diapause *aak-1/2* L4 hermaphrodites were crossed with healthy nonstarved *aak-1/2* males, and the reciprocal cross was also performed. The resulting F_1 brood size was evaluated and the percentage of parents that exhibit a reduced brood size was calculated from plates with successful crosses. A minimum of 40 successful crosses was analyzed. To determine the ability of wild-type gene copies to rescue observed reproductive defects in our transgenerational rescue experiments, L4 hermaphrodites from late-generation (F_7 or F_8) post-L1 diapause *aak-1/2* mutants that exhibited severe brood-size defects were crossed with healthy nonstarved wild-type or *aak-1/2* males. Reproductive defects were determined in the resulting cross progeny, as described above.

RNAi Soaking During the L1 Diapause. dsRNAs that corresponded to *set-2*, *set-16*, *ash-2*, *wdr-5*, *par-5*, and GFP (as a control), were prepared as described previously (58). Wild-type and *aak-1/2* L1 larvae were obtained by alkaline hypochlorite treatment and were maintained in M9 buffer containing dsRNA of the query gene (1–1.5 μg/μL) during the 3-d diapause. L1 larvae were then transferred to seeded NGM plates to recover to the L4 stage. Subsequently, 50 larvae were collected for each dsRNA tested, maintained at 20 °C for 3 or 4 d, after which their fertility and brood size were scored. Adults were sterile or fertile, whereas fertile animals could either be "reduced brood size" (<100 F_1), or "normal brood size" (>100 F_1). For transgenerational analysis (from F_1 to F_3), 50 F_1 L4-stage larvae obtained from parents with reduced brood size were singled to seeded NGM plates and their fertility and brood size were scored as above. Each experiment was performed three independent times.

DRB Treatment. We determined our working concentration of 30 μM DRB through a typical LE50 drug dosage. Wild-type and *aak-1/2* L1 larvae obtained by alkaline hypochlorite treatment, were maintained in M9 buffer containing 30 μM DRB, or not, during the 3-d duration in the diapause. Following the starvation, postdiapause L1 larvae were transferred to seeded NGM plates and when they reached the L4 stage 50 larvae per condition were isolated and incubated at 20 °C for 3 or 4 d to assess their fertility and brood size. The reproductive defects fall into three different categories: sterile, fertile with reduced brood size (≤100 F_1), or fertile with normal brood size (>100 F_1). For transgenerational analysis (from F_1 to F_3), 50 F_1 animals at the L4 stage, obtained from parents with reduced brood size, were singled onto NGM plates seeded with OP50, and their fertility and brood size were scored as described above. Each experiment was performed minimally three independent times.

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