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Suspended Animation Extends Survival Limits of *Caenorhabditis elegans* and *Saccharomyces cerevisiae* at Low Temperature

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The orderly progression through the cell division cycle is of paramount importance to all organisms, as improper progression through the cycle could result in defects with grave consequences. Previously, our lab has shown that model eukaryotes such as *Saccharomyces cerevisiae*, *Caenorhabditis elegans*, and *Danio rerio* all retain high viability after prolonged arrest in a state of anoxia-induced suspended animation, implying that in such a state, progression through the cell division cycle is reversibly arrested in an orderly manner. Here, we show that *S. cerevisiae* (both wild-type and several cold-sensitive strains) and *C. elegans* embryos exhibit a dramatic decrease in viability that is associated with dysregulation of the cell cycle when exposed to low temperatures. Further, we find that when the yeast or worms are first transitioned into a state of anoxia-induced suspended animation before cold exposure, the associated cold-induced viability defects are largely abrogated. We present evidence that by imposing an anoxia-induced reversible arrest of the cell cycle, the cells are prevented from engaging in aberrant cell cycle events in the cold, thus allowing the organisms to avoid the lethality that would have occurred in a cold, oxygenated environment.

INTRODUCTION

The cell division cycle is an intricate series of interrelated events that must occur in a tightly regulated manner during each iteration of the cycle in order to maintain high fidelity (Alberts *et al.*, 2007). Abnormal progression through the cell cycle could have dire consequences, such as chromosome missegregation, aneuploidy, or cell death. To help maintain the fidelity of the cycle, organisms have evolved cell cycle checkpoint mechanisms that function to prevent cells from engaging in improper progression through the cycle (Hartwell and Kastan, 1994). While these checkpoint mechanisms are often sufficient to safeguard cell division cycle fidelity, the function of these checkpoints can be compromised by various combinations of genetic mutation and adverse environmental conditions (e.g., Moir and Botstein, 1982).

Many laboratories have used a combination of mutations and environmental manipulations to study the consequences of the cell cycle gone awry (e.g., Hartwell *et al.*, 1970; Nurse and Thuriaux, 1980; Moir and Botstein, 1982; Toda *et al.*, 1983). Perhaps the most celebrated of these efforts were the pioneering studies carried out by Hartwell and colleagues, using temperature-sensitive (ts) mutants in the budding yeast *Saccharomyces cerevisiae* that exhibited cell division cycle (cdc) phenotypes when shifted to the restrictive temperature (Hartwell *et al.*, 1970). While this work yielded many insights into the workings of the cell cycle, other labs took a complementary approach using cold-sensitive (cs) mutants in the hopes of identifying additional genes that are involved in controlling cell cycle progression. Botstein and colleagues found many cs mutants in *S. cerevisiae* that exhibited characteristic arrest at specific points in the cell cycle when shifted to the cold (Moir *et al.*, 1982). Thus, in budding yeast, considerable work has been done to examine the interaction of lowered temperature with certain genetic backgrounds that result in cell cycle defects.

Similarly, our lab is interested in the response of model organisms to environmental stresses and has previously shown that the model eukaryotes *S. cerevisiae* (Chan and Roth, 2008), *Caenorhabditis elegans* (Padilla *et al.*, 2002), and *Danio rerio* embryos (Padilla and Roth, 2001) all enter into a reversible state of profound hypometabolism when subjected to extreme oxygen deprivation. We call this phenomenon anoxia-induced suspended animation, as all life processes that can be observed by light microscopy reversibly arrest, pending restoration of oxygen. Moreover, because these model eukaryotes retain high viability, it is probable that complex processes, such as progression through the cell cycle, are reversibly halted in an orderly manner. For example, the *san-1* (suspended animation-1) gene, encoding a component of the spindle checkpoint, is required for *C. elegans* embryos to engage in anoxia-induced suspended animation (Nystul *et al.*, 2003). As such, we sought to determine if we could exploit this conserved phenomenon of orderly cell cycle arrest to enhance survival of model systems that are prone to cell cycle errors when subjected to an environmental insult, in this case, lowered temperatures.

In this work, we report that wild-type *C. elegans* embryos are unable to survive a 24-h exposure to 4°C. This lethality is associated with extensive chromosome segregation defects in the cold embryos. Similarly, we show that cold-sensitive mutants of *S. cerevisiae*, as well as the wild-type strain

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BY4741 (the parental strain for the *MAT**a*** deletion set), all can be made to exhibit cold-sensitive lethality associated with abnormal cell cycle progression when grown at low temperature. Further, we find that when these organisms are placed into a suspended state, they are protected from cold-induced insults and retain high viability, at least partly because they are prevented from proceeding through the cell cycle in an error-prone manner.

MATERIALS AND METHODS

Yeast Strains and Environments

Yeast strains DBY640 (*MAT**a*** gal$^-$ *mal*$^-$ *ade2*), DBY1252 (*MAT**a*** *ade2 cdc51-1*), DBY1583 (*MAT**a*** *his4-539 ura3-52 lys2-801 ndc1-1*), and DBY1802 (*MAT**a*** *his3-524-539 ura3-52 lys2-801*) were generous gifts from Dr. David Botstein (Princeton University). *cin1*Δ and *cin2*Δ were constructed de novo in the BY4741 (*MAT**a*** *his3*Δ*1 leu2*Δ*0 met15*Δ*0 ura3*Δ*0*) background and verified by PCR using standard methods (http://labs.fhcrc.org/gottschling/Yeast%20Protocols/index.html). Anoxic atmospheres were generated by either of two means: continuous perfusion in modified Pyrex crystallization dishes with nitrogen gas (N_2; Air Gas Nor Pac, Seattle, WA) scrubbed with an Aeronex CE500KF14R inline gas purifier to remove residual oxygen or using Bio-Bag Environmental Chamber Type A (Becton Dickinson, Franklin Lakes, NJ) per manufacturer's instructions. Both methods yielded equivalent results. Yeast were placed into one of several VWR Low Temperature Incubators Model No. 2005 (VWR, West Chester, PA) that were set to the appropriate temperature for cold exposures. Temperature was verified at the beginning and end of each experiment using an alcohol thermometer immersed in a beaker of water that was thermally equilibrated within the incubator.

Yeast Immunofluorescence

To obtain yeast for cell biological analysis, cells were grown overnight in 5 ml YPD. Cells were collected by light centrifugation, washed once in PBS, and then resuspended in a small volume of PBS. A small portion of the cells were plated at low density onto solid YPG media (1% yeast extract, 2% peptone, 3% glycerol, with benomyl where appropriate) to assess viability by colony formation. (Glycerol, i.e., nonfermentable, media was used in all yeast experiments in order to enable the yeast to undergo suspended animation in anoxia.) The remaining cells were split evenly and plated onto sterile nylon membranes (GE Water and Process Technologies, Trevose, PA) on YPG plates. One set of plates was made anoxic at low temperature, whereas the controls were incubated at the same low temperature in room air.

After the appropriate incubation periods, the cells were collected by centrifugation in PBS. Cells were fixed with 3.7% formaldehyde for 1 h at room temperature, followed by two washes using 100 mM potassium phosphate buffer, pH 7.5, and one wash using the same buffer supplemented with 1.2 M sorbitol. Cell walls then were digested using standard methods to form spheroplasts. Spheroplasts were applied to poly-L-lysine–treated slides and allowed to settle. After removing excess liquid, slides were incubated for 5–6 min in methanol at −20°C, followed by 30 s in acetone at room temperature. Slides were washed three times using PBS with 1% BSA. Slides were then incubated overnight at room temperature in the presence of primary antibody mAb YL1/2 against α-tubulin (Abcam, Cambridge, MA) at 1:250 dilution in PBS-BSA. Slides were washed four times with PBS-BSA before application of secondary antibody (anti-rat conjugated AlexaFluor 488) at 1:250 dilution, with incubation for 2 h at room temperature. Slides were washed four times with PBS-BSA, twice more with PBS, and stained with DAPI for 5 min before mounting in 1 mg/ml phenylenediamine, pH 9, in 90% glycerol. Images were acquired using a Zeiss MRm camera on a Zeiss Axioskop with AxioVision 4.6 software (Thornwood, NY).

Nematode Viability in Hypothermic and Anoxic Environments

As a population source for all experiments, wild-type Bristol strain N2 was continuously maintained at 20°C with care taken to ensure that the population never starved (Brenner, 1974). For experiments concerning embryos laid on a plate, five adults were allowed to lay eggs for 2 h in a spot of *Escherichia coli* (OP50) on a small nematode growth medium (NGM) plate. The adults were removed and the plate with embryos placed into the appropriate environment. In all viability experiments, the nematodes were allowed to recover at 20°C in room air, after exposure to the cold. Embryos were scored for hatching 24-h after exposure, and then followed to adulthood. Animals that could not be accounted for were not included in the total.

For experiments with two-cell embryos, adult *C. elegans* were picked into a drop of sterile water containing 100 μg/ml ampicillin, 15 μg/ml tetracycline, and 200 μg/ml streptomycin on a glass plate. Adults were chopped with a razor blade, and two-cell embryos were transferred by mouth pipette. Thirty to 60 two-cell embryos were transferred to a small glass boat (custom-made to fit atmospheric chambers; Avalon Glass Works, Seattle, WA) filled with 3 ml of 1% agarose in M9 buffer. The glass boats were then placed in glass syringes for exposure to the appropriate environment. After exposure, agarose chunks containing the embryos were cut out of the boat and placed with embryos facing up onto a medium-sized NGM plate seeded with OP50. Embryos were scored for hatching 24-h after exposure, and hatched larvae were transferred to the surface of the plate and followed to adulthood. Animals that could not be accounted for were not included in the total.

Oxygen deprivation experiments were performed as described previously (Padilla *et al.*, 2002; Nystul and Roth, 2004). To generate anoxic environments we used either an Anaerobic Bio-Bag Type A Environmental Chamber according to the manufacturer's instructions, or perfused an environmental chamber with 100% N_2 gas at 80 ml/min. The chambers were designed by Dr. Wayne Van Voorhies (New Mexico State University, Las Cruces, NM; Van Voorhies and Ward, 2000). The environmental chamber is a 30-ml glass syringe (Popper and Sons, New Hyde Park, NY) fitted with a custom steel stopper lined with two Viton O-rings to ensure a tight seal. The stopper is bored through and has a steel luer lock on the exterior face so that a hose carrying compressed gas can be attached. A desired gas mixture is delivered to the chamber at a constant pressure and flow rate from compressed tanks by passing first through a rotometer (Aalborg, Orangeburg, NY) or a mass flow controller (Sierra Instruments, Monterey, CA) to monitor flow rate and then through a 250-ml gas washing bottle (Kimble Chase, Vineland, NJ) containing 250 ml of water to hydrate the gas. A one-eighth–inch outer diameter nylon or fluorinated ethylene propylene tubing (Cole-Parmer, Vernon Hills, IL) was used, and connections between tubing and regulators were made with brass John Guest-type fittings (Airgas, Nor Pac, Vancouver, WA). All other connections were made with either microflow quick-connect fittings or standard luer fittings (Cole-Parmer).

To generate reproducible hypothermic environments, we calibrated a dedicated incubator (Low Temperature Incubator VWR Model No. 2005 or Echotherm [Torrey Pines Scientific, San Diego, CA] Chilling Incubator IN35) for each experiment. Temperature measurements were continuously monitored and logged for confirmation using HOBO data loggers (Onset, Pocasset, MA).

Nematode Immunofluorescence

To generate slides enriched for young embryos (chromosomes and microtubule-organizing centers (MTOCs) are large and easy to see in young embryos), a synchronous population of young adults was generated by bleaching (Epstein and Shakes, 1995). At the onset of egg laying, the synchronous adults were washed off and chopped with razor blades on a glass plate. The minced material was spread evenly on an NGM plate and exposed to the appropriate environment as described. After exposure, the embryos were washed off the plate in water, concentrated, and frozen onto slides using dry ice (Moore *et al.*, 1999). Slides were frozen within 4 min of removing the embryos from the exposure environment.

To examine chromosome morphology, coverslips were flicked off the frozen slides and embryos fixed in prechilled methanol for 5 min at −20°C. Slides were rinsed twice in PBS and then placed in PBS-BSA for 30 min before incubating with mAb YL1/2 and mAb 414 (specific for the nuclear pore complex, Abcam) for 1 h at room temperature. The YL1/2 and 414 antibodies were used at 1:1000 and 1:500 dilution in PBS-BSA, respectively. Slides were washed three times in blocking solution and then incubated with secondary antibodies for 1 h at room temperature. Secondary antibodies (goat anti-rat Alexa Fluor 488 and goat anti-mouse Alexa Fluor 568) were diluted 1:200 in PBS-BSA. Slides were rinsed two times in PBS-BSA and a third time in PBS-BSA containing DAPI to stain DNA. For consistency, only the AB to ABa and ABp division was scored for missegregation phenotypes. This comprises the two- to three-cell transition in embryogenesis. All data points are the result of at least three independent slide preparations.

To score MTOC duplication events, slides were prepared as described and fixed in −20° methanol for 5 min. Primary antibody mAb YL1/2 and secondary antibody goat anti-rat 488 were used at the same dilutions as above. For each environmental condition and time point, the number of MTOCs in each blastomere was scored. Only embryos with two to six blastomeres were scored. All data points are the result of at least three independent slide preparations.

Images were acquired using a DeltaVision multiple wavelength fluorescent microscope (Applied Precision, Issaquah, WA). Stacks of 0.2-μm optical sections were collected for the indicated wavelengths and then deconvolved using Softworx software (Applied Precision). All nematode images in this report are two-dimensional projections of multiple optical sections.

RESULTS

Prolonged Exposure to Cold Temperature Results in Lethality for the cdc51-1 and ndc1-1 Mutants in Yeast

To begin our studies, we sought to identify environmental conditions in which model eukaryotes exhibit lethality that is correlated with dysregulation of the cell cycle, with the idea that rescue from such lethality might be possible by imposing anoxia-induced suspended animation. Accord-

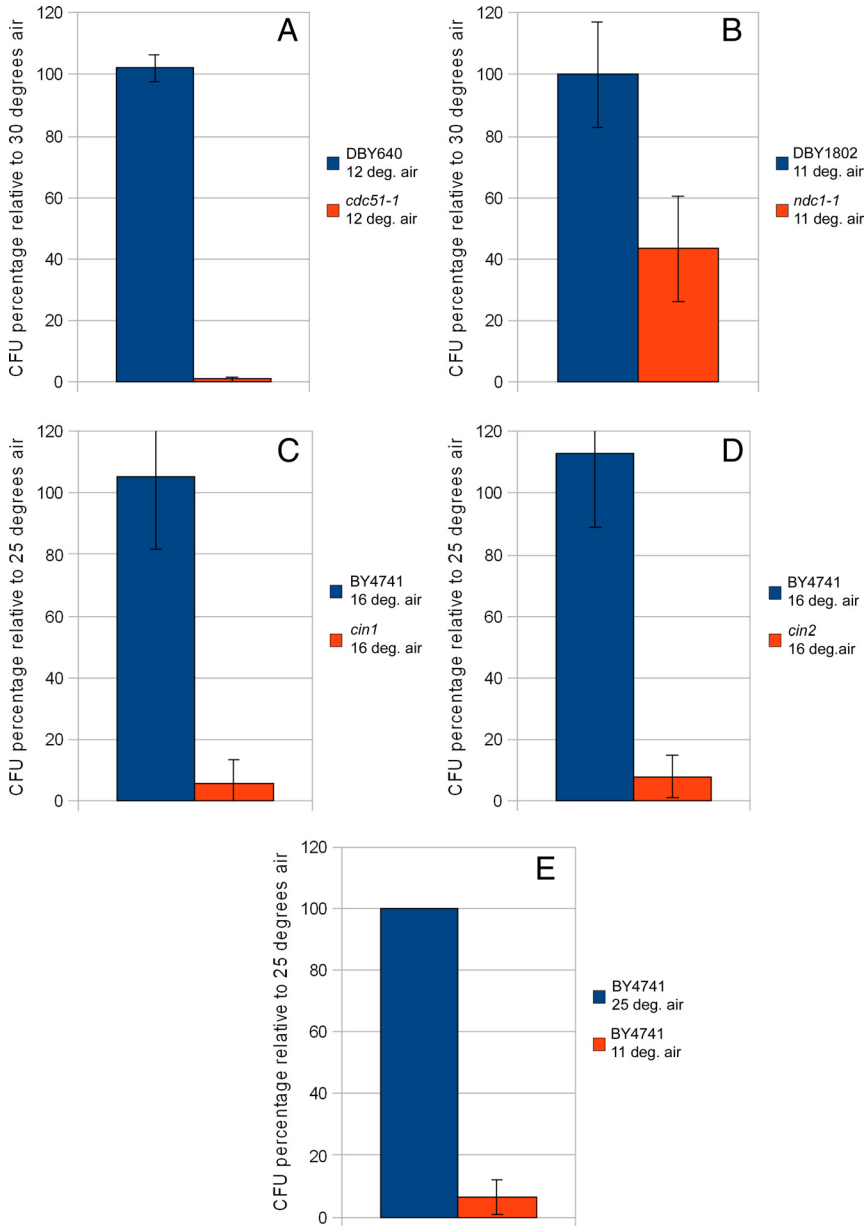

Figure 1. Prolonged exposure to low temperature causes defects in subsequent colony formation in four mutant strains, as well as the wild-type BY4741 strain. Error bars in all figures indicate 1 SD from the mean. (A) *cdc51-1* and DBY640 parental strain control after 4 d at 12°C on YP glycerol media. (B) *ndc1-1* and DBY1802 parental strain after 7 d at 11°C on YP glycerol. (C) *cin1*Δ and BY4741 parental strain after 7 d at 16°C on YP glycerol with 1.0 μg/ml benomyl. (D) *cin2*Δ and BY4741 parental strain after 4 d at 16°C on YP glycerol with 1.0 μg/ml benomyl. (E) BY4741 after 7 d at 25 and 11°C on YP glycerol with 10 μg/ml benomyl. For both Figures 1 and 5, after each respective cold exposure, the plates were returned to permissive conditions to allow recovery for at least 1 wk before the counting of colonies. For both figures, data were derived from five or six independent trials for each genotype.

ingly, we obtained a number of cold-sensitive cell cycle mutants in budding yeast from the Botstein lab and tested these strains to determine whether they were cold-sensitive for survival, as assayed by the ability to form colonies. Two such strains, DBY1252 and DBY1583, which bear the *cdc51-1* (Moir *et al.*, 1982) and the *ndc1-1* (Thomas and Botstein, 1986) mutations, respectively, manifested such a conditional viability defect. *cdc51-1* and *ndc1-1* cells formed colonies as well

Table 1. *cdc51-1* cells at 12°C tended to accumulate as large-budded cells with an undivided single nucleus that is far from the bud neck. This defect was prevented in *cdc51-1* cells that were made cold while anoxic. For Tables 1 through 4, comparing numbers of normal cells to aberrant cells between the two mutant populations results in Fisher's exact test; $p < 0.0001$. Values in parentheses are percentages.

	DBY640 day 4 air 12°C (n = 363)	*cdc51-1* day 4 air 12°C (n = 398)	DBY640 day 4 anoxia 12°C (n = 366)	*cdc51-1* day 4 anoxia 12°C (n = 255)
Normal unbudded cells	271 (74.7)	186 (46.7)	320 (87.4)	209 (80.4)
Normal small-budded cells	41 (11.3)	4 (1.0)	18 (4.9)	21 (8.1)
Normal large-budded cells	51 (14.0)	21 (5.3)	27 (7.4)	17 (6.7)
Large-budded cells, aberrant nuclear configuration	0	184 (46.2)	0	5 (1.9)
Other (aberrant) cells	0	3 (0.8)	1 (0.3)	3 (1.2)

as their respective parental strains (DBY640 and DBY1802) on rich medium with glycerol as the sole carbon source (a nonfermentable medium) at 30°C. In contrast, when *cdc51-1* cells were shifted to 12°C for 4 d after plating, there was a 100-fold decrease in the ability to form colonies, despite being shifted back to 30°C to allow growth of any surviving cells at day 4 (see Figure 1A). Similarly, *ndc1-1* cells that were shifted to 11°C for 7 d after plating showed a 60% decrease in colony formation, after allowing for recovery after the cold exposure (see Figure 1B). Neither parental strain exhibited a colony forming defect after experiencing the same shift to low temperature.

Prolonged Exposure to Low Temperature in the Presence of Benomyl Results in Lethality for cin1Δ and cin2Δ Mutants, as well as the Wild-Type Strain BY4741

We also examined two deletion mutants that Botstein and colleagues had identified as cold-sensitive, *cin1*Δ and *cin2*Δ (Hoyt *et al.*, 1990). These two mutants formed small colonies that were comparable in number to the BY4741 parental strain when shifted to a range of cold temperatures after plating on rich medium with glycerol as the sole carbon source (data not shown). However, we noted that *cin1*Δ and *cin2*Δ were also sensitive to the microtubule-depolymerizing drug benomyl (Stearns *et al.*, 1990). It is possible then that benomyl and low temperature would act additively to disrupt microtubule function more than low temperature alone. We therefore determined whether it were possible to elicit a colony-forming defect similar to *cdc51-1* and *ndc1-1*, by spotting *cin1*Δ and *cin2*Δ cells onto media with a range of benomyl concentrations and exposing these cells to various cold temperatures. Using this approach and follow-on colony formation assays, we found that *cin1*Δ cells showed a 90% decrease in colony formation when incubated at 16°C for 7 d on plates with 1.0 μg/ml benomyl (see Figure 1C). Similarly, *cin2*Δ cells showed an >80% decrease in colony formation after 4 d at 16°C on plates with 1.0 μg/ml benomyl (see Figure 1D). This concentration of benomyl was well tolerated by these mutants at a permissive temperature (25°C, see Supplemental Figure 1), but was lethal at the restrictive temperature.

Given our success at inducing a colony forming defect in *cin1*Δ and *cin2*Δ using low temperature combined with benomyl, we tested whether a similar effect could be obtained for an essentially wild-type strain, BY4741. After testing a range of benomyl concentrations combined with a range of low temperatures, we found that BY4741 cells showed a 90% decrease in colony formation on media with 10 μg/ml benomyl after a 7-d exposure to 11°C (see Figure 1E). In total, we have identified two mutants (*cdc51-1* and *ndc1-1*) that exhibit a cold-sensitive colony forming defect, another two mutants (*cin1*Δ and *cin2*Δ) that show a similar defect in the presence of low doses of benomyl, and a wild-type strain (BY4741) that manifests the cold-sensitive colony forming defect in a higher dose of benomyl.

The Cold-sensitive Lethality in Yeast Is Associated with Abnormal Cellular Morphology That is Consistent with Errors in Cell Cycle Progression

Botstein and colleagues had previously reported that *cdc51-1* (Moir *et al.*, 1982) and *ndc1-1* (Thomas and Botstein, 1986), as well as *cin1*Δ and *cin2*Δ (Hoyt *et al.*, 1990) all showed abnormal cell cycle progression at low temperature, as revealed by staining for DNA and tubulin. We likewise used DAPI and the anti-tubulin antibody YL1/2 to determine if similarly abnormal cell morphology occurred in the cells we had treated with low temperature. Previously, *cdc51-1* cells were reported to tend to arrest as large-budded cells with undivided nuclei when shifted to 17°C (Moir *et al.*, 1982). We verified this observation, albeit using a longer exposure to compensate for the slower growth rate on the nonfermentable glycerol medium, and at a slightly lower temperature of 12°C (see

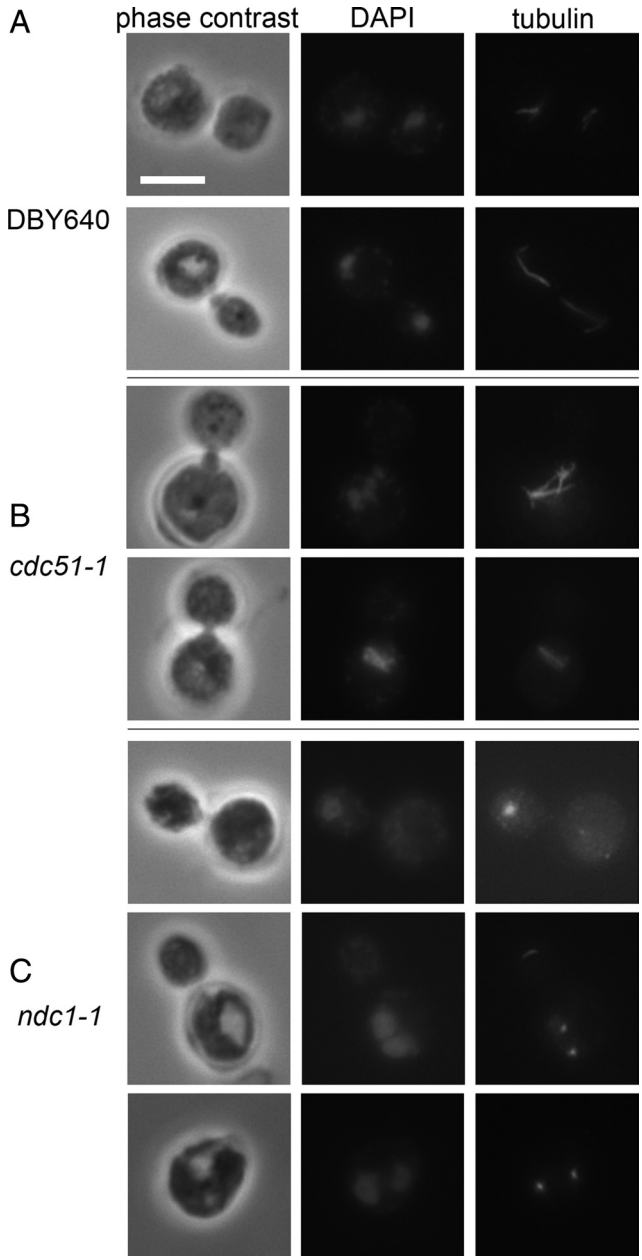

Figure 2. Typical cell biological abnormalities observed in various yeast strains in the cold. Scale bar, 5 μm. (A) Wild-type nuclear and tubulin configurations in DBY640. Other parental strains showed similar patterns (not shown). (B) *cdc51-1* cells in the terminal large-budded state with undivided nuclei and abnormal tubulin configurations. (C) *ndc1-1* cells showing a range of phenotypes. The top cell has a single undivided nucleus in a large-budded cell that is far from the bud neck. The middle cell is a large-budded cell with two nuclei in the mother cell. The bottom cell is unbudded, yet has two nuclei. In cells with double nuclei, each nucleus is typically in proximity to a focus of tubulin staining, presumptive spindle pole bodies. The multiple nuclei phenotype that is relatively prevalent in *cin1*Δ, *cin2*Δ, and BY4741 cold-aerated cells is the same as in *ndc1-1* and is omitted for brevity.

Table 2. Similar to *cdc51-1*, *ndc1-1* cells at 11°C tended to accumulate as large-budded cells with single undivided nuclei far from the bud neck. There was also a smaller increase in the incidence of cells with multiple nuclei. These defects were prevented in *ndc1-1* cells that are made cold while anoxic. Values in parentheses are percentages.

	DBY1802 11°C air (n = 278)	*ndc1-1* 11°C air (n = 350)	DBY1802 11°C anoxia (n = 269)	*ndc1-1* 11°C anoxia (n = 310)
Normal unbudded cells	249 (89.6)	187 (53.4)	227 (84.4)	262 (84.5)
Normal small-budded cells	10 (3.6)	6 (1.7)	18 (6.7)	20 (6.5)
Normal large-budded cells	18 (6.5)	21 (6.0)	22 (8.2)	18 (5.8)
Large-budded cells, aberrant nuclear configuration	1 (0.4)	97 (27.7)	1 (0.4)	0
Multiple or fragmented nuclei	0	28 (8.0)	1 (0.4)	8 (2.6)
Aploid	0	11 (3.1)	0	0
Other (aberrant)	0	0	0	2 (0.7)

Table 1). After 4 d at 12°C, 46.2% of *cdc51-1* cells exhibited the characteristic terminal large-budded cell phenotype (Moir *et al.*, 1982), with an undivided nucleus in one of the cell bodies, far from the bud neck (see Table 1). In contrast, none of the large-budded cells in the parental strain showed any discernible abnormalities.

Reminiscent of cold-sensitive tubulin mutants (Huffaker *et al.*, 1988), we found that in almost all cases, microtubules were only found in the nucleated cell within a large-budded pair of the *cdc51-1* strain; the other cell would be devoid of immunoreactivity as well as DAPI staining (see Figure 2B). This result was never observed in the parental strain (see Figure 2A) and provides an explanation for the undivided nucleus phenotype, as proper chromosome segregation and nuclear division should be impossible given such abnormal spindle orientations.

Similar to *cdc51-1*, *ndc1-1* previously has been shown to accumulate as large-budded cells with undivided nuclei after shifting to 13°C, accompanied by abortive cell divisions that give rise to a significant population of cells with no discernible nuclear DNA (called aploid cells,Thomas and Botstein, 1986). At 11°C on glycerol media, we found a similar increase in the percentage of large-budded cells with undivided nuclei (27.7% of total cells examined, see Table 2), as well as an increase in the percentage of cells with either multiple or fragmented nuclei (8.0%) and aploid cells (3.1%). In total, 38.9% of cold-treated *ndc1-1* cells showed obviously abnormal nuclear morphology, whereas only one out of the 278 parental strain cells we examined showed such a defect. Consistent with the nuclear morphology defect, tubulin staining in the *ndc1-1* abnormal large-budded cells almost always consisted of a single tubulin aster near the single undivided nucleus, with an absence of staining in the anucleate cell body (see Figure 2C). In binucleate *ndc1-1* cells, each nucleus was similarly accompanied by a tubulin aster (see Figure 2C).

Previously, both *cin1*Δ and *cin2*Δ were shown to accumulate as large-budded cells after shifting to 11°C, with most of these cells exhibiting the single undivided nucleus phenotype (Hoyt *et al.*, 1990). In our hands, *cin1*Δ cells that were incubated for 7 d at 16°C in the presence of 1.0 μg/ml benomyl on glycerol showed an enrichment for cells with multiple or fragmented nuclei (34%), whereas such abnormal cells were not observed in the BY4741 parental strain (see Table 3). Similarly, *cin2*Δ cells that were incubated for 4 d at 16°C in 1.0 μg/ml benomyl showed an increase in the percentage of cells with multiple or fragmented nuclei (24.5%, see Table 4). In contrast, all of the examined BY4741 control cells were morphologically normal under the same incubation conditions.

Finally, we determined if BY4741 cells incubated under conditions that result in a cold-sensitive colony formation defect (7 d at 11°C on 10 μg/ml benomyl) also showed abnormal nuclear and tubulin morphology. Surprisingly, these cells only manifested a mild increase in the percentage of morphologically abnormal cells: 21 of 236 cells examined (8.9%) had multiple or fragmented nuclei, 1 of 236 was aploid, and 1 of 236 was a small-budded cell with a single nucleus in the bud (see Table 5). However, considering that the cold-sensitive colony formation defect in BY4741 is an effect of large magnitude, we consider it likely that some of the cold-induced cell cycle defects are more subtle than that which can be observed by examining gross nuclear DNA and tubulin morphology.

A 24-h Exposure to 4°C Causes Lethality in the Embryos of C. elegans That Is Associated with Multiple Types of Cell Cycle Defects

To determine if a similar phenomenon could be observed in a metazoan model system, we examined the embryos of wild-type N2 *C. elegans* exposed to cold temperatures. Similar to the yeast, >99% of *C. elegans* embryos exposed to 4°C

Table 3. *cin1*Δ cells at 16°C in the presence of 1.0 μg/ml benomyl showed an increased proportion of cells with multiple or fragmented nuclei. This defect was prevented in *cin1*Δ cells that are made cold while anoxic. Values in parentheses are percentages.

	BY4741 16°C air (n = 353)	*cin1*Δ 16°C air (n = 203)	BY4741 16°C anoxia (n = 178)	*cin1*Δ 16°C anoxia (n = 225)
Normal unbudded cells	335 (94.9)	125 (61.6)	172 (96.6)	210 (93.3)
Normal small-budded cells	3 (0.8)	0	1 (0.6)	2 (0.9)
Normal large-budded cells	15 (4.2)	8 (3.9)	3 (1.7)	13 (5.8)
Large-budded cells, aberrant nuclear configuration	0	1 (0.5)	0	0
Multiple or fragmented nuclei	0	69 (34.0)	2 (1.1)	0
Aploid	0	0	0	0
Other (aberrant)	0	0	0	0

Table 4. *cin2*Δ cells at 16°C in the presence of 1.0 μg/ml benomyl showed an increased proportion of cells with multiple or fragmented nuclei. This defect was prevented in *cin2*Δ cells that are made cold while anoxic. Values in parentheses are percentages.

	BY4741 16°C air (n = 353)	*cin2*Δ 16°C air (n = 326)	BY4741 16°C anoxia (n = 178)	*cin2*Δ 16°C anoxia (n = 280)
Normal unbudded cells	335 (94.9)	238 (73.0)	172 (96.6)	270 (96.4)
Normal small-budded cells	3 (0.8)	0	1 (0.6)	2 (0.7)
Normal large-budded cells	15 (4.2)	7 (2.1)	3 (1.7)	6 (2.1)
Large-budded cells, aberrant nuclear configuration	0	0	0	0
Multiple or fragmented nuclei	0	80 (24.5)	2 (1.1)	2 (0.7)
Aploid	0	1 (0.3)	0	0
Other (aberrant)	0	0	0	0

for 24-h died as embryos or shortly after hatching into larvae (see Figure 3A). A more detailed accounting of developmental progression after cold exposure can be found in Supplemental Figure 2. We found that 78% of such embryos contained blastomeres that manifested profound chromosome segregation defects when examined after cold exposure (see Figure 3B). The most frequent of these defects were the "cut" phenotype, where the cleavage furrow is apparently bisecting the mitotic DNA mass (33%, see Figure 3C), and multiple nuclei (29%, see Figure 3E). In addition, we observed blastomeres either with multipolar spindles, anaphase bridging (see Figure 3D), or containing no nuclear DNA, at low frequency. Although a few embryos managed to complete embryogenesis, most of these died as young larvae, suggesting that prolonged exposure to low temperature during early embryogenesis had profoundly detrimental effects on the animals' continued development.

Because low temperatures near the freezing point for water are known to cause the depolymerization of microtubules (Osborn and Weber, 1976), we visualized microtubule configuration using the YL1/2 antibody (see Figure 4). As expected, antibody staining revealed two MTOCs within each dividing blastomere in control two-cell embryos (see Figure 4A) maintained at 20°C. In contrast, embryos exposed to 4°C often contained blastomeres that possessed various numbers of excessive MTOCs (see Figure 4, B, C, and D). The severity of this ectopic MTOC phenotype is correlated with the duration of exposure to the cold. After 4 h at 4°C, <5% of blastomeres had at least five MTOCs. By 14 h at 4°C, 65% of blastomeres had at least five MTOCs, while 17% of blastomeres had at least 10. After 24-h at 4°C, over 80% of blastomeres had at least five MTOCs, whereas over 35% had at least 10 MTOCs (Figure 4E). Taken together, these results suggest that low temperature causes dysregulation of the cell cycle and a striking manifestation of such dysregulation is that the process of MTOC duplication becomes uncoupled from other aspects of the cycle, e.g., cytokinesis.

Table 5. BY4741 at 11°C in the presence of 10 μg/ml benomyl showed an increased incidence of cells with multiple or fragmented nuclei. This defect was prevented in cells made cold while anoxic. $p < 0.0001$ by Fisher's exact test, comparing total numbers of normal and aberrant cells. Values in parentheses are percentages.

	BY4741 11°C air (n = 236)	BY4741 11°C anoxia (n = 244)
Normal unbudded cells	206 (87.3)	238 (97.5)
Normal small-budded cells	3 (1.3)	0
Normal large-budded cells	4 (1.7)	2 (0.8)
Large-budded cells, aberrant nuclear configuration	0	0
Multiple or fragmented nuclei	21 (8.9)	4 (1.6)
Aploid	1 (0.4)	0
Other (aberrant)	1 (0.4)	0

Anoxia-induced Suspended Animation Enables Survival of Otherwise Lethal Cold Exposure in Yeast

Given that the cold-induced lethality we observed is associated with defects in progression through the cell cycle and that we had previously shown that anoxia-induced suspended animation reversibly halts the cell division cycle, we reasoned that placing the two model systems into a suspended state might preserve viability by imposing an orderly arrest of the cycle. Indeed, we found that making the *cdc51-1* cells anoxic on glycerol medium at 12°C resulted in a substantial rescue of the cold-induced lethal phenotype. Although <1% of cold room air cells survived to form colonies, 66% of cold anoxic cells successfully formed colonies upon being shifted back into permissive conditions (see Figure 5A). We observed a similar rescuing effect for the other four genotypes as well. For *ndc1-1*, cells exposed to 11°C in air formed colonies at 43% of the capacity of permissive temperature controls, whereas cells exposed to 11°C in anoxia retained 83% colony forming capacity (see Figure 5B). *cin1*Δ cells exposed to 16°C in air in the presence of 1.0 μg/ml benomyl formed colonies at only 6% of the capacity of controls, in contrast to cold anoxic cells that retained 59% colony forming capacity (see Figure 5C). *cin2*Δ cells exposed to 16°C in air in the presence of 1.0 μg/ml benomyl formed colonies at 8% of the capacity of controls, compared with 71% in anoxia-treated cells (see Figure 5D). Finally, BY4741 cells exposed to 11°C in air on 10 μg/ml benomyl formed colonies at 6% of the capacity of controls, whereas cells exposed to cold in anoxia retained 58% of colony-forming capacity (see Figure 5E). These data show that, when yeast from a variety of genotypes are exposed to cold temperatures (with or without benomyl), i.e., conditions that cause significant lethality, it is possible to prevent much of this lethality by imposing a state of anoxia-induced suspended animation. This rescuing effect of anoxia was quite robust, with paired Student's *t* test $p < 0.005$ when comparing cold-aerated with cold anoxic cells, in all cases.

Anoxia-induced Suspended Animation Prevents Yeast from Accumulating Lethal Cell Cycle Errors during Cold Exposure

To determine whether anoxia-induced suspended animation can impose a relatively orderly arrest of the cell cycle that prevents cold yeast cells from committing irrecover-

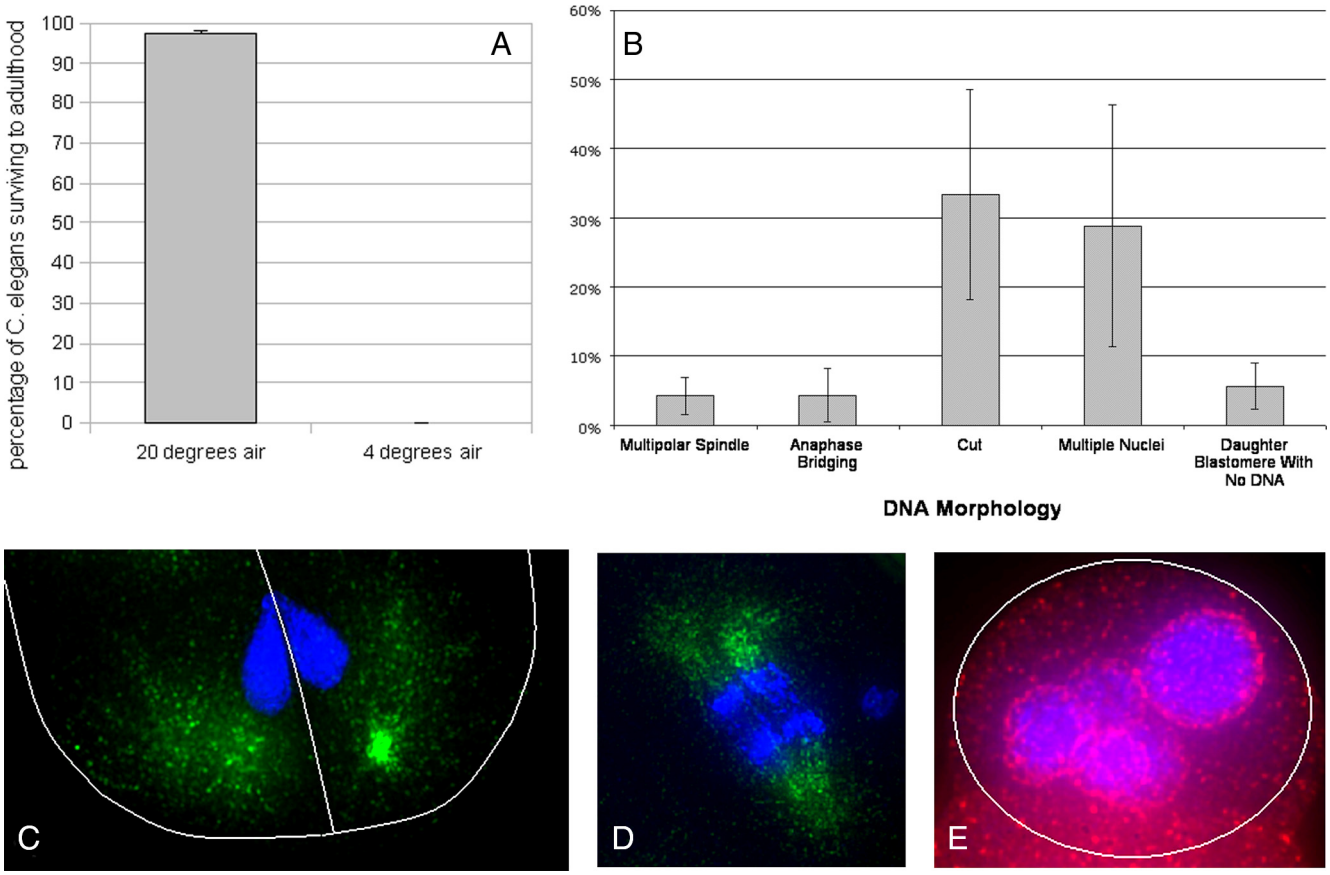

Figure 3. *C. elegans* embryos exposed to 4°C for 24-h exhibit high lethality that is associated with defects in the cell cycle. (A) Almost none of the embryos so exposed survive to adulthood. In fact, the vast majority fail to complete embryogenesis. (B) Quantification of the incidence of various cell cycle defects seen in such embryos. (C) The most commonly seen defect is the incomplete separation of two blastomeres by the cleavage furrow, called the cut phenotype. DNA in blue, tubulin in green. (D) An example of a blastomere exhibiting anaphase bridging, i.e., attachment of microtubules from both MTOCs to a single chromosome that results in mechanical breakage of the DNA when chromosome segregation occurs. (E) A blastomere with multiple nuclei. Nuclear pore complex staining in red.

able errors in cell cycle progression, we compared the cellular morphology of cold anoxic cells to that of cold-aerated cells. In all five genotypes examined, there is a large decrease in the percentage of cells with abnormal morphology within the population that underwent cold exposure while anoxic. This decrease is accompanied by a relative increase in the percentage of (normal) unbudded cells. For *cdc51-1*, 46.2% of cold-aerated cells showed the characteristic terminal-arrest phenotype, whereas only 1.9% of cold-anoxic cells showed the same morphology. Conversely, only 46.7% of cold-aerated cells had a normal unbudded morphology, whereas 80.4% of cold-anoxic cells were in this state (see Table 1). *ndc1-1* showed a very similar pattern, with a large decrease in the percentage of abnormal large-budded cells (27.7% in cold-aerated conditions to none in cold anoxia) and a large increase in the percentage of unbudded cells (53.4–84.5%, see Table 2). *cin1*Δ showed a large decrease in the percentage of cells with multiple or fragmented nuclei (34% in cold-aerated conditions to none in cold anoxia) that is nearly matched by a large increase in the percentage of unbudded cells (61.6–93.3%, see Table 3). Similar trends were observed in *cin2*Δ (Table 4) and BY4741 (Table 5). However, since the prevalence of the cold-induced morphological defects in these two backgrounds is lower, the magnitude of the rescuing effects are also necessarily smaller. These data suggest that imposing a state of anoxia-induced suspended animation on yeast in the cold prevents the cells from attempting to divide in an error-prone manner that contributes to the lethality observed under cold, oxygen-replete conditions.

Anoxia-induced Suspended Animation Prevents the Accumulation of Lethal Cell Cycle Errors and Enables Survival of Nematode Embryos Exposed to 4°C

Similar to the yeast, placing nematode embryos into anoxia-induced suspended animation before cold exposure protected them against cold-induced lethality. Ninety-seven percent of the anoxic embryos survived the 4°C exposure, to successfully develop to adulthood (see Figure 6A and compare with Figure 3A). In addition, similar to the yeast, the blastomeres in the nematode embryos were prevented from progressing improperly through the cell cycle. Consistent with this idea, none of the cell cycle defects we observed in room air 4°C embryos were seen in the suspended embryos (see Figure 6B). We also found that the order of exposure to cold temperature and anoxia has an important effect on the likelihood of survival (see Figure 6C). Specifically, two-cell embryos that were first transitioned into anoxia before the temperature shift successfully completed em-

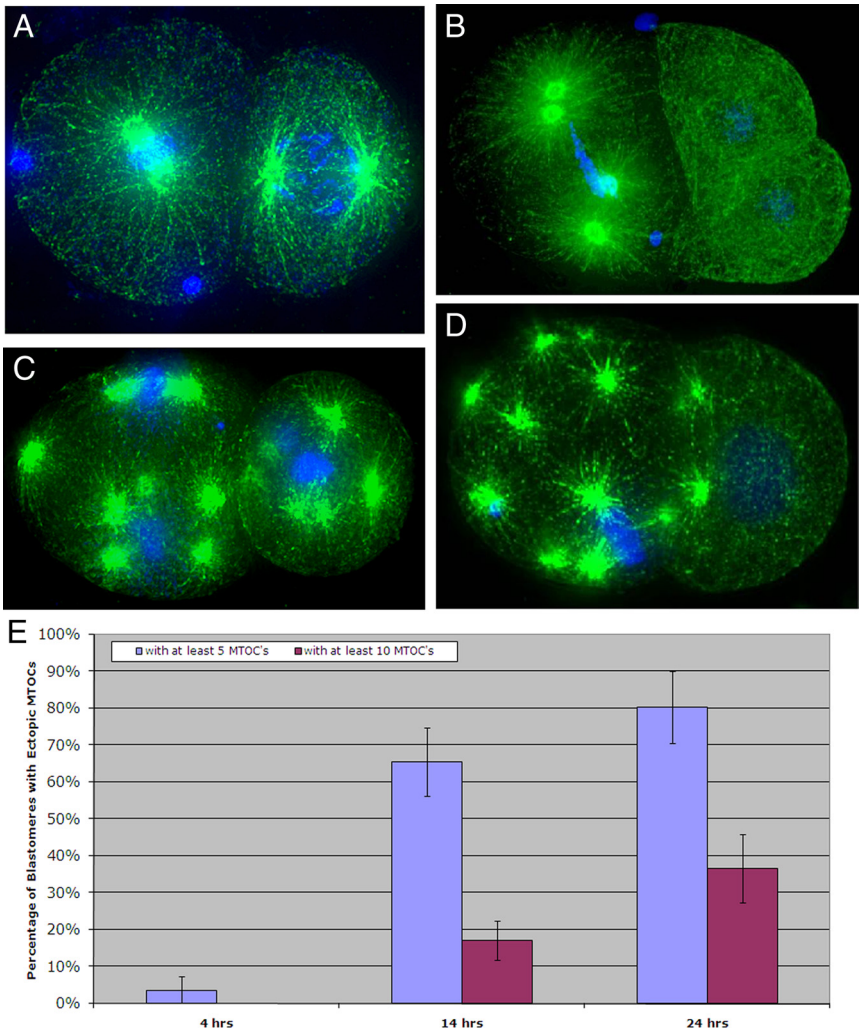

Figure 4. Excessive MTOC duplication in *C. elegans* embryos exposed to the cold. Embryos are ~50 μm in length. DNA staining in blue, tubulin in green, anterior to the left. (A) A control 20°C two cell embryo with two MTOCs per blastomere. (B) After a four hour exposure to 4°C, the anterior blastomere in this embryo has four MTOCs. (C) Similarly, after a 14 h exposure, the anterior blastomere in this embryo has eight MTOCs while the posterior blastomere has five. (D) After a 24-h exposure, the anterior blastomere of this embryo has 12 MTOCs. (E) The number of MTOC duplication events increases over time during cold exposure. Only blastomeres containing greater than two MTOCs were scored. Each data point is the result of at least three independent slide preparations.

bryogenesis >85% of the time. In contrast, two-cell embryos that were shifted into the cold before the onset of anoxia successfully completed embryogenesis at a rate of only 21%, suggesting that proper entry into the suspended state before cold challenge is necessary to retain high survivability. Thus, we have shown that anoxia-induced suspended animation can preserve the potential for life in nematode embryos that are faced with otherwise lethally cold temperatures.

DISCUSSION

Anoxia-induced Suspended Animation Prevents Yeast Cells from Committing Irrecoverable Cell Cycle Errors at Low Temperatures

In this article, we report that several mutant strains in *S. cerevisiae* that were previously reported to be cold sensitive (*cdc51-1*, *ndc1-1*, *cin1*Δ, and *cin2*Δ), as well as the wild-type strain BY4741, can be made to manifest irrecoverable cell cycle defects that are associated with a high incidence of lethality after prolonged exposure to low temperature. Such phenotypes had previously been noted in these four mutants (Moir *et al.*, 1982; Thomas and Botstein, 1986; Hoyt *et al.*, 1990), which is consistent with the fact that the gene products mutated in these mutants are associated with tubulin function. Ndc1 has a role in spindle pole body duplication (Chial *et al.*, 1999), whereas Cin1 and Cin2 are both involved in the proper folding of β-tubulin (Hoyt *et al.*, 1997). It is thus not surprising that these mutants are more sensitive to low temperature than wild type, as microtubule dynamics is itself a cold-sensitive process (Osborne and Weber, 1976). Although strain BY4741 is much less sensitive to low temperature (the addition of 10 μg/ml benomyl is required to sensitize this strain to low temperature), we nonetheless observed similar cell cycle errors in this wild-type strain. This result suggests that perhaps *all* yeast strains can be similarly rendered cold-sensitive, given the "right" combination of low temperature and drug concentration to induce irreversible cell cycle errors due to impaired tubulin function.

We also found that when these same strains are made cold under anoxic conditions on nonfermentable media, the cold-induced lethality is diminished to a degree that is highly statistically significant. That is, many more cells retain the ability to form colonies after the cold exposure, provided they were kept in an anoxic state in the cold. Whereas cold, oxygen-replete cells showed obvious cell cycle defects, including undivided nuclei in large-budded cells, multiple nuclei within the same cell, and cells devoid of nuclear DNA, these defects were almost completely abolished in

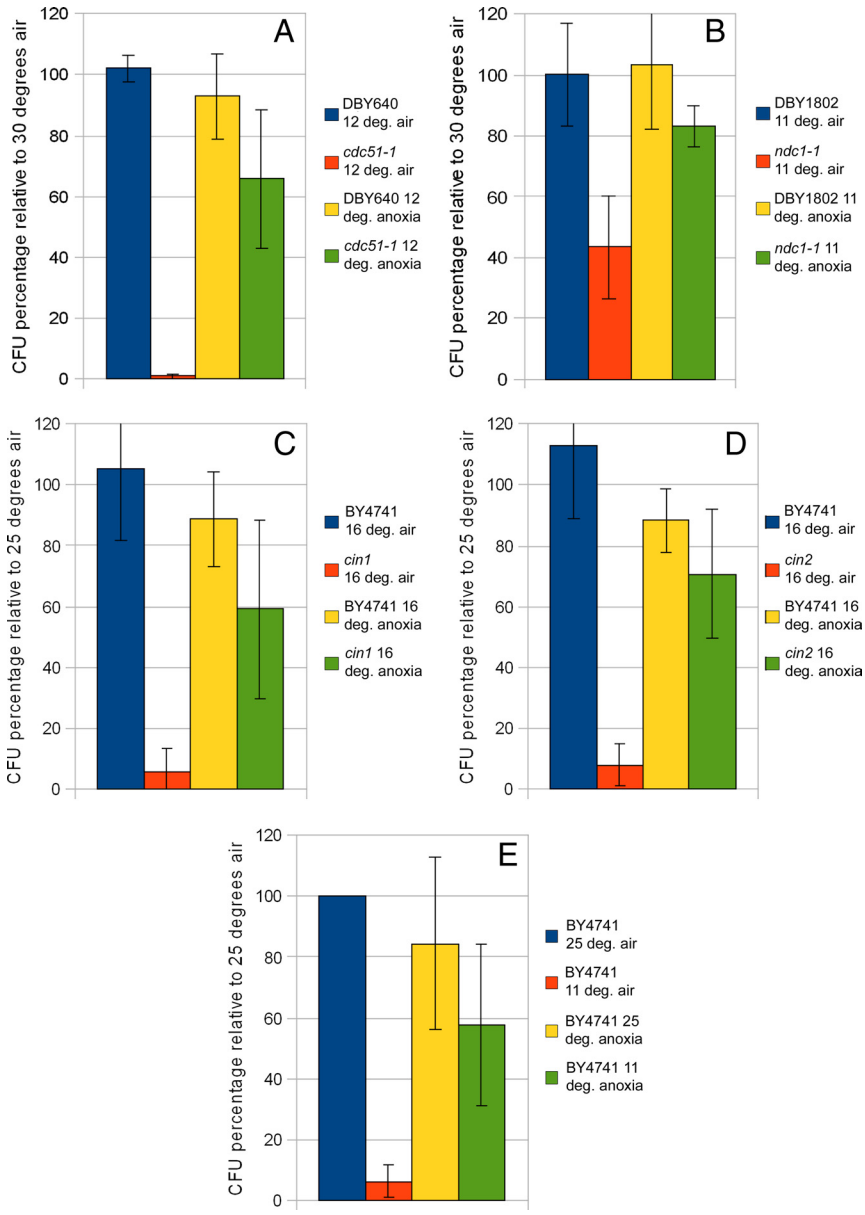

Figure 5. Anoxia-induced suspended animation rescues yeast cells from cold lethality. Data are shown for each of the following genotypes after cold exposure either in room air or in anoxia. (A) *cdc51-1* and DBY640 parental strain control after 4 d at 12°C on YP glycerol media. (B) *ndc1-1* and DBY1802 parental strain after 7 d at 11°C on YP glycerol. (C) *cin1*Δ and BY4741 parental strain after 7 d at 16°C on YP glycerol with 1.0 μg/ml benomyl. (D) *cin2*Δ and BY4741 parental strain after 4 d at 16°C on YP glycerol with 1.0 μg/ml benomyl. (E) BY4741 after 7 d at 25°C and 11°C on YP glycerol with 10 μg/ml benomyl. $p < 0.005$ by paired Student's *t* test for each comparison between cold-aerated and cold-anoxic mutant cells (A–D) or between cold-aerated and cold-anoxic BY4741 cells (E).

cold, anoxic cells. These results are consistent with the idea that when the cells are put into a state of anoxia-induced suspended animation, their cell cycles are arrested in an orderly, reversible manner. This prevents the occurrence of irrecoverable cell cycle errors, as revealed by examination of DNA and tubulin morphology as well as assays for colony formation. On return to permissive growth conditions, the cells that were suspended in anoxia are shown to be protected from the cold lethality, whereas cells that were cold, but not suspended, show much higher lethality. As we noted, a wild-type strain can be made cold sensitive by the addition of a microtubule-destabilizing agent, and this cold sensitivity can be substantially rescued by anoxia-induced suspended animation. If it were possible for all yeast strains to be rendered susceptible to cell cycle error-driven cold lethality by an appropriate concentration of an agent like benomyl, our results suggest that, in general, it might be possible to prevent such cold lethality by imposing a suspended state in anoxia.

Anoxia-induced Suspended Animation Similarly Protects C. elegans Embryos from Cold Lethality by Preventing Excessive MTOC Duplication

Similar to the yeast, nearly all wild-type *C. elegans* embryos that are exposed to 4°C for 24-h fail to survive to adulthood, despite being returned to normal growth temperatures after the bout of cold. In fact, most never complete embryogenesis. Cell biological analysis of cold two-cell embryos showed that a variety of cell biological defects occur at low temperature. These defects include the cut phenotype, multiple nuclei, blastomeres with no DNA, anaphase bridging, and multipolar spindles. Instead of a uniform arrest at a specific point in the cell cycle, there is apparently a combination of different defects in nuclear division and chromosome segregation that result in a variety of terminal phenotypes. Thus, low temperature is a particularly destructive stress to young nematodes, as the cold almost invariably terminally disrupts embryogenesis or subsequent development in a number of ways.

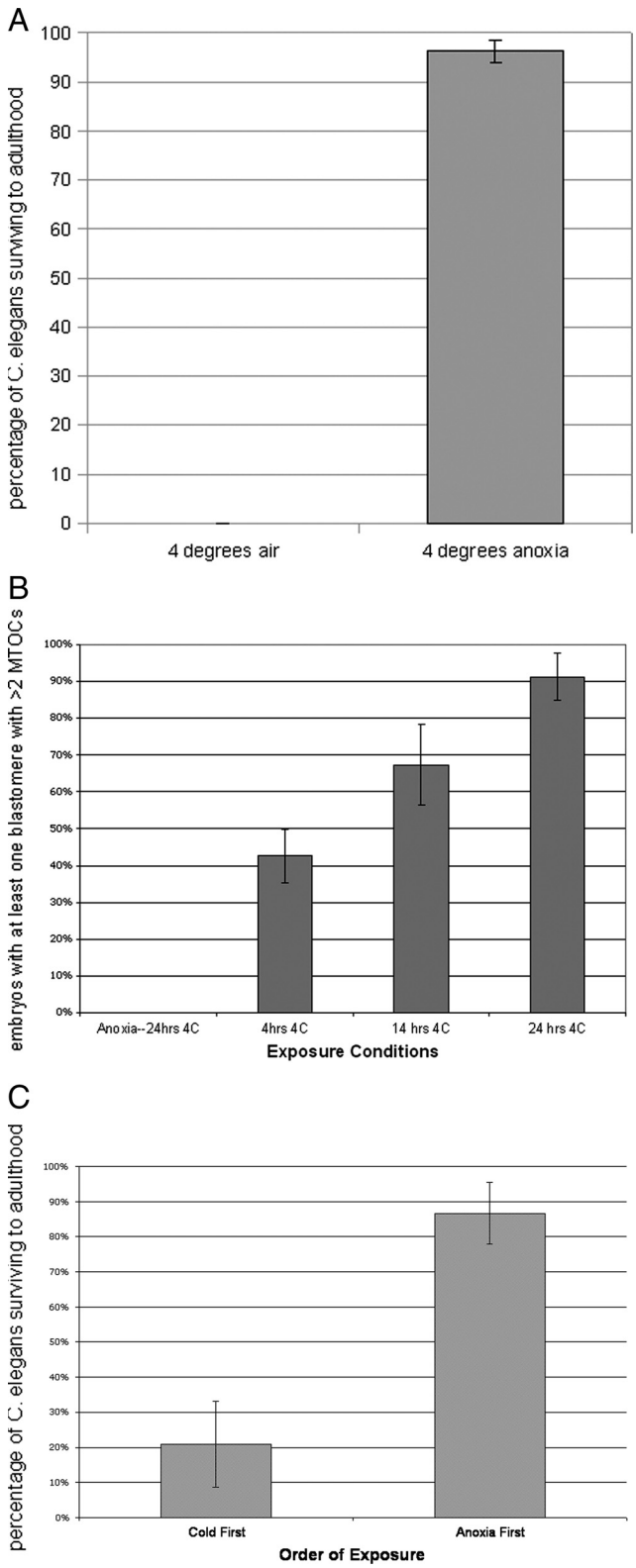

Figure 6. Anoxia-induced suspended animation almost completely rescues nematode embryos from cold-induced lethality. (A) In contrast to embryos that spent 24-h at 4°C in room air, almost all embryos that were suspended in anoxia before transition to the cold survived to reach adulthood after being shifted back to 20°C. (B) Embryos suspended in anoxia do not exhibit MTOC reduplication errors. (C) Eighty-seven percent of two-cell embryos that are first transitioned into anoxia before cold exposure survive, but only 21% of embryos first transitioned into the cold before anoxia survive, indicating that establishment of the suspended state before temperature transition is required to attain the full protective effect. Data from at least three independent trials.

Perhaps our most striking cell biological observation is the increased incidence of excessive MTOCs with increasing duration of cold exposure. At four hours in the cold, 43% of embryos are already affected, as these embryos contain at least one blastomere with an excessive MTOC. By 24-h, over 90% of embryos are similarly affected. Also, the numbers of excessive MTOCs increases dramatically over time, such that by 24-h in the cold, 80% of blastomeres have at least five MTOCs, whereas 37% have at least 10 MTOCs. Not surprisingly, the occurrence of excessive MTOCs coincides with aberrant DNA configurations that presage the eventual death of the cold-exposed embryos, because the subcellular architecture has been hopelessly distorted. The profound perturbations in tubulin configuration also help to explain the various other cell biological phenotypes that were already mentioned.

The manifold reduplication of MTOCs in cold embryos suggest that the cold induces a loss of synchronization between MTOC duplication and other aspects of the cell cycle, e.g., cytokinesis. One possible explanation for this result is that MTOC duplication is relatively less energetically costly than cytokinesis, such that in an energy-limited state in the cold, MTOC duplication continues to occur while there is insufficient energy to carry out cytokinesis. An alternative explanation is that, in the cold, there exist active mechanisms that are sufficient to arrest the majority of processes that comprise cell cycle progression (in an orderly manner), but MTOC duplication is not among the processes that are so governed. Our experiments do not address this issue, but it is thought that the maintaining of proper control of MTOC duplication is intimately linked to orderly progression of the cell division cycle (Azimzadeh and Bornens, 2007). We propose that quite independently of the questions addressed in this article, the application of our methodology to induce desynchronization of the centrosome cycle might be exploited to further elucidate the molecular mechanisms associated with this crucial component of the cell cycle.

We found that nematode embryos that are kept in an anoxic, suspended state in the cold do not reduplicate their MTOCs, thus avoiding this irrecoverable type of cell cycle error. As a result, when the nematodes are returned to permissive growth conditions, the anoxic, cold embryos survive while the vast majority of oxygenated, cold embryos do not complete embryogenesis and virtually none of these ultimately progresses to adulthood. These results are consistent with the view that by imposing anoxia, the embryos are unable to generate sufficient energy to attempt MTOC duplication in the cold. In effect, synchronization of the cell cycle is maintained in the suspended state because all processes that we can observe by microscopy have been reversibly and uniformly halted. This forced maintenance of cell cycle synchrony results in much higher survivorship compared with embryos where synchrony is not maintained.

Anoxia-induced Suspended Animation Is Likely to Be Protective against Relatively Subtle Cell Cycle Errors Caused by Low Temperature

Although a striking correlation is observed between the prevention of aberrant cell cycle progression and im-

proved survival following anoxia in the cold, we note that not all of the yeast or nematode embryos exposed to the cold in room air manifested discernible cell cycle defects. Yet the large majority of these yeast or nematodes fail to survive. Thus, cold-induced damage can be subtle, but still lethal. For example, while examination of nuclear DNA by DAPI staining can reveal gross abnormalities such as multiple nuclei, more subtle defects, such as mis-segregation of single chromosomes, would not be detectable. Considering our results, we conclude that the suspended state in anoxia probably prevents many different forms of cold-induced damage, whether they be subtle or gross. Collectively, the prevention of these many possible errors helps to enable survival in otherwise lethally cold temperatures.

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