Multiple Isoforms of CDC25 Oppose ATM Activity to Maintain Cell Proliferation during Vertebrate Development

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Abstract

The early development of vertebrate embryos is characterized by rapid cell proliferation necessary to support the embryo’s growth. During this period, the embryo must maintain a balance between ongoing cell proliferation and mechanisms that arrest or delay the cell cycle to repair oxidative damage and other genotoxic stresses. The ataxia-telangiectasia mutated (ATM) kinase is a critical regulator of the response to DNA damage, acting through downstream effectors, such as p53 and checkpoint kinases (CHK) to mediate cell-cycle checkpoints in the presence of DNA damage. Mice and humans with inactivating mutations in ATM are viable but have increased susceptibility to cancers. The possible role of ATM in limiting cell proliferation in early embryos has not been fully defined. One target of ATM and CHKs is the Cdc25 phosphatase, which facilitates cell-cycle progression by removing inhibitory phosphates from cyclin-dependent kinases (CDK). We have identified a zebrafish mutant, standstill, with an inactivating mutation in cdc25a. Loss of cdc25a in the zebrafish leads to accumulation of cells in late G2 phase. We find that the novel family member cdc25d is essential for early development in the absence of cdc25a, establishing for the first time that cdc25d is active in vivo in zebrafish. Surprisingly, we find that cell-cycle progression in cdc25a mutants can be rescued by chemical or genetic inhibition of ATM. Checkpoint activation in cdc25a mutants occurs despite the absence of increased DNA damage, highlighting the role of Cdc25 proteins to balance constitutive ATM activity during early embryonic development. Mol Cancer Res; 10(11); 1451–61. ©2012 AACR.

Introduction

During the early phases of vertebrate embryo development, rapid cell proliferation is required to support the growth of the embryo and organogenesis. Throughout this time, the developing embryo is exposed to a variety of genotoxic stressors. External stressors include environmental toxins and chemicals as well as radiation, both ionizing and ultraviolet (1). Several endogenous sources of DNA damage have also been recognized, including oxidants, estrogens, alkylators, and the products of lipid peroxidation and metabolic pathways (2, 3). Failure to repair the damage due to these extrinsic and intrinsic agents can lead to mutations that contribute to cancer, aging, and degenerative diseases (1, 4). In response to genotoxic stress, cells have evolved robust networks to detect DNA damage and to arrest or delay cell-cycle progression, allowing time for repair of the damage (5, 6). Testifying to the importance of these mechanisms, humans or mice with germline mutations in DNA damage response genes display a range of developmental defects, including growth retardation, microcephaly, cerebellar ataxia, sterility, bone marrow failure, and immunodeficiency as well as predisposition to cancer (1, 4). Among the best characterized of these networks is the response to double-strand DNA (dsDNA) breaks, which is coordinated by the phosphoinositide-3-kinase–related protein kinase (PIKK) family member, ataxia-telangiectasia mutated (ATM; ref. 7). Upon sensing a dsDNA break or other change in chromatin structure, ATM phosphorylates key downstream targets including p53 and the checkpoint kinase 1 (CHK1) and checkpoint kinase 1 (CHK2), which in turn bring about cell-cycle arrest or delay to allow for repair through the homologous recombination or nonhomologous end joining pathways, or alternatively apoptosis and cell death (7–9). While ATM has clearly been shown to be important for the response to exogenous DNA damage, its possible roles in normal development are less clear. Similar to humans, mice deficient in ATM are viable but display growth retardation, neurologic dysfunction, infertility, defective T-lymphocyte maturation, radiation sensitivity, and increased cancer incidence (10, 11). Exposure to teratogens, such as phenytoin or genotoxic insults, such as gamma radiation, impairs the development.
of ATM-null embryos, and simultaneous deletion of ATM and other DNA damage repair factors including histone H2AX and DNA-dependent protein kinase is embryonic lethal in mice (12, 13). However, owing to the relative inaccessibility of mammalian embryonic development, it is unclear what roles ATM may play during the course of normal embryogenesis. In particular, it is not known to what degree ATM-dependent checkpoint mechanisms may oppose cell proliferation during early embryogenesis.

One of the key targets of the ATM-CHK1/2 network is the CDC25 family of phosphatases, key regulators of the cell cycle. Under conditions that favor cell-cycle progression, CDC25 removes inhibitory phosphates from cyclin-dependent kinases (CDK), allowing cyclin/CDK complexes to drive cell-cycle transitions (14). In response to dsDNA breaks, CHK1 and CHK2 phosphorylate CDC25, targeting it for destruction and impeding CDK activation (15). Mammalian cells express 3 different CDC25 genes: CDC25A, CDC25B, and CDC25C. CDC25A seems to act alone in controlling entry from G1 to S and intra-S progression, whereas all 3 CDC25 genes function in G2–M progression (16).

The developmental roles of Cdc25 have been partially defined. In mice, Cdc25A is essential for embryonic development. Cdc25A−/− embryos are resorbed at around E6.5 due to widespread apoptosis (17). Although Cdc25A−/− mice die early in development, mice lacking Cdc25B and Cdc25C survive with normal cell-cycle progression and checkpoint function (18). Therefore, Cdc25A likely compensates for the other Cdc25 genes and may be the most functionally important mammalian Cdc25. Unlike mammals, zebrafish express a single canonical CDC25, designated cdc25a, during embryogenesis (19). In zebrafish embryos, overexpression of cdc25a drives cells into mitosis (19) and blocks cell-cycle lengthening and acquisition of G2 phase as early embryonic cell cycles give rise to postmitiblastula transition, asynchronous cell cycles (20). Zebrafish also express a divergent family member, designated cdc25d, which is homologous to cdc25a but is not present in mammals (19). cdc25d can rescue a fission yeast cdc25a mutant, but has not been shown to have detectable activity in zebrafish (19, 20). It is not known whether the single canonical zebrafish cdc25a is essential for development, or whether cdc25a and cdc25d have any redundant roles in cell-cycle regulation, nor is it known if zebrafish cdc25 family members participate in DNA damage checkpoints.

Previously, we conducted a screen for mutations that affect embryonic cell proliferation in zebrafish (21). Here, we report the identification of an inactivating mutation in zebrafish cdc25a. cdc25a is essential for zebrafish embryonic development, and cdc25a-deficient mutants accumulate cells in G2–M phase. The cell-cycle defects of cdc25a-deficient mutants can be partially rescued by cdc25d, showing in vivo activity of this divergent family member. We reasoned that this model could help us to understand epistatic relationships of CDC25 to other checkpoint genes in a whole organism that is not subject to extrinsic genotoxic stress. We find that chemical or genetic inhibition of ATM rescues the accumulation of cells in G2–M phase in cdc25a-deficient embryos. ATM is activated in the cdc25a mutants despite the absence of widespread DNA double-strand breaks (DSB), and we present evidence that ATM also impedes cell-cycle progression in embryos with wild-type levels of CDC25 activity. These results emphasize the important balance between mechanisms that favor cell proliferation and the ATM-mediated checkpoint response during early embryonic development in vertebrates.

**Materials and Methods**

**Fish maintenance**

Zebrafish were maintained according to standard procedures (22). All work with zebrafish was carried out under protocols approved by the Institutional Animal Care and Use Committees at University of Texas Southwestern Medical Center (Dallas, TX), an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-accredited institution.

**Zebrafish immunohistochemistry and immunofluorescence**

Twenty-four-hour-old embryos were dechorionated, euthanized with tricaine, and fixed in 4% paraformaldehyde (PFA) in 1× PBS overnight at 4°C. Immunohistochemistry (IHC) was conducted using 1:1,000 anti–phosphohistone H3 Serine 10 (pH3; Santa Cruz Biotechnology, catalog no. sc-8656-R); 1:200 anti-mouse Cdc25A (Santa Cruz Biotechnology, catalog no. sc-97) or 1:1,000 zebrafish-specific anti–phosphoHistone H2AX (23) followed by incubation with 1:350 horseradish peroxidase–conjugated goat anti-rabbit immunoglobulin G (IgG; Jackson Immunoc antibod icals) and staining with diaminobenzidine (DAKO) for 10 minutes according to the manufacturer’s protocol. For fluorescent imaging, secondary antibody was a 1:15,000 dilution of Alexafluor-488–conjugated goat anti-rabbit IgG (Invitrogen). Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay was conducted using the Apoptag Red In Situ Apoptosis Detection Kit (Millipore) as described in ref. (24). Acridine orange staining was conducted as described in ref. (24). Immunoblots were conducted with rabbit anti–phospho-CHK1 (Ser835) from Cell Signaling Technology. The antibody recognizes a protein of 50 kD in lysates of zebrafish embryos, in good agreement with the predicted size of zebrafish chk1 (410 amino acids; Accession: NP_956487.1) and was previously used to detect activated chk1 protein in lysates of zebrafish tissue (25). Anti–phospho-Rb (Ser 795) is validated for recognition of phosphorylated zebrafish retinoblastoma (Rb) protein (www.cellsignal.com/products/9301.html) and was previously shown to recognize phospho-Rb in zebrafish (26).

**Comet assay**

Twenty-four or 48-hour zebrafish embryos were disaggregated to a single-cell suspension as described in ref. (27). Zebrafish cells were embedded in low-melt agarose gel, lysed, and then electrophoresed using the Trevigen CometAssay Kit according to the manufacturer’s protocol (Trevigen).
Drug treatment

Twelve-hour–old embryos were treated with 10 μmol/L ATM kinase inhibitor KU55933 (Calbiochem) in E3 zebrafish embryo medium (22) and 1% dimethyl sulfoxide (DMSO) for 12 hours at 28°C, then euthanized with tricaine, fixed in 4% PFA/1× PBS overnight at 4°C, and processed for IHC. To quantify anti-pH3 staining, 12 images were taken for each treatment and genotype. The pH3-positive cells in an area extending from the end of the yolk tube extension to the tip of the tail were independently counted for each embryo by 2 observers who were blinded to the identity of the samples. All counts were normalized by the total area (measured using ImageJ software) to account for differing size of the tail regions.

Cell culture and transfection

The human non–small-cell lung cancer cell line A549 (kindly provided by Dr. J. Minna, UT Southwestern Medical Center, Dallas, Texas) was maintained in RPMI media supplemented with 10% FBS. For overexpression of Cdc25, cells were transfected with pCMV-SPORT6 containing a full-length Mus musculus Cdc25A cDNA (Accession number BC046296, Open Biosystems) with 1:1,000 pS1981-ATM (Rockland) or 1:500 anti-H2AX (Millipore) as described in ref. (28).

Morpholino injections

A total of 0.5 mmol/L Cdc25a 5′UTR (TAATCAGCCGGCGGATTAAGAAG), cdc25a splice site (ATGACACCTCACCTAGCCATGTT), control (CCTCTTCCTACCTCGGTGAC), or cdc25d 5′UTR (AAATGACACCTCACCTAGCCATGTT) morpholinos were injected into 1 to 2 cell zebrafish embryos from the AB strain or from offspring of cdc25a+/− adults. Morpholinos were purchased from Gene-Tools. ATM, CDKN1A, and ATR morpholinos were as described in ref. (23,29). The genotypes of injected embryos were confirmed by PCR using primers flanking the cdc25a mutation site (GGTTGTTCGACTCCATCTCGT and CACAGCAGACGGCTAACG) followed by digestion of the PCR product with BsiEI (New England Biolabs) and gel electrophoresis.

Results

The standstill mutation causes embryonic cell-cycle accumulation in the G2 phase of the cell cycle

Previously, we described a forward-genetic screen to identify mutants that altered embryonic cell proliferation in the haploid F2 progeny of ENU-mutagenized zebrafish (21, 30). Using whole-mount IHC with an antibody specific for the phosphorylated form of histone H3 (to evaluate cell proliferation), 7 mutant lines were identified including slyez1, sfdcz215, mybl2ez226, espl1ez280, llgeez322, sdcx319, and sdbez1 (21). The standstill mutant (sdz) was identified on the basis of a markedly decreased fraction of pH3-positive cells in the mutant embryos (Fig. 1). The standstill mutant morphologic phenotype becomes evident at approximately 24 hours postfertilization (hpf) and includes microcephaly, microphthalmia, abnormal body shape, and reduced pigmentation (Supplementary Fig. S1). To further understand the cell proliferation defect, we conducted flow cytometric DNA content analysis, which showed an accumulation of cells in the G2–M phase of the cell cycle (Fig. 1E). Histone H3 is phosphorylated in late G2 phase, coincident with the onset of chromatin condensation, and remains phosphorylated until the chromosomes begin to decondense in anaphase (31, 32). Thus, the standstill mutation seems to result in accumulation of cells in the G2 phase of the cell cycle, before the onset of chromatin condensation (33).

Positional cloning of the standstill gene

To understand the molecular nature of the defect in standstill embryos, we used meiotic recombinational mapping to identify the mutant locus. We conducted bulk segregant analysis (34) followed by intermediate-resolution...
mapping with a panel of 88 mutant embryos to assign standstill to zebrafish chromosome 13. For high-resolution mapping, we assembled a panel of 1,758 phenotypically mutant embryos and localized the mutation to an approximately 5 cM interval between microsatellite markers Z6259 and ZJA5 (Fig. 2). The map position was further refined with a combination of known markers and novel microsatellites derived from genomic sequence. The critical interval contains a single gene, the zebrafish ortholog of Cdc25A (19). We sequenced the cdc25a gene from wild-type and standstill mutant embryos and found that the mutants contain a C to T mutation at codon 206 of the coding sequence, which is predicted to result in a premature stop codon and a truncated protein lacking the catalytic domain (Fig. 2B and C). standstill mutant embryos did not stain with an anti-mouse Cdc25A antibody (Fig. 2E), and whole-mount in situ hybridization revealed reduced cdc25a mRNA in standstill mutants, consistent with nonsense-mediated decay (Supplementary Fig. S2).

To confirm that cdc25a deficiency was responsible for the standstill phenotype, we designed a morpholino oligonucleotide (MO) targeting the splice junction between exon 1 and intron 1 of the cdc25a pre-mRNA. cdc25a MO-injected embryos displayed a phenotype identical to the standstill mutant, including microcephaly, microphthalmia, and bent tail. Immunohistochemical staining for pH3 showed that the MO-injected embryos had reduced cell proliferation, phenocopying the standstill mutation (Fig. 2G). We obtained identical results with a translation-blocking MO directed against the cdc25a initiating ATG (Supplementary Fig. S3). Thus, standstill is encoded by zebrafish cdc25a.

cdc25d cooperates with cdc25a during embryonic development

During the cell cycle, Cdc25 removes inhibitory phosphates from CDKs to allow cell-cycle progression. Despite lacking a key cell-cycle regulator, cdc25a−/− embryos undergo gastrulation and relatively normal organogenesis. The ability of embryos to complete early stages of development

Figure 2. standstill encodes a zebrafish Cdc25 ortholog. Positional cloning of standstill (A). Bulk segregant analysis assigned standstill to zebrafish chromosome 13. Further analysis of 3,456 meioses using microsatellite markers narrowed the critical interval to a 0.1 cM region containing cdc25a. Microsatellite markers are prefixed with the letter “z.” CT573293 and BX571943 are 2 adjoining genomic contigs in zebrafish genome assembly Zv9. Arrows indicate the orientation of predicted genes. B and C, electropherograms of fragment of cdc25a exon 7 from wild-type (wt; B) and standstill mutant (C) embryos, showing the codon 206 C-T mutation generating a nonsense allele in the mutant. D and E, whole-mount IHC of wt (D) and standstill/cdc25a−/− (E) embryos stained with anti-mouse Cdc25A antibody. The mutant embryos have diminished anti-Cdc25A reactivity. F and G, knockdown of cdc25a phenocopies the standstill phenotype. F, injection of wt embryos with a control MO did not affect cell proliferation. G, injection of a splice site MO targeting the cdc25a pre-mRNA exon 1/intron 1 boundary caused a marked reduction in the number of pH3-positive cells.
could be due in part to residual maternally supplied wild-type cdc25a. However, early development also occurs in embryos injected with translation-blocking MO (Supplementary Fig. S3), which is predicted to inhibit translation of both maternal and zygotic mRNAs. We investigated whether cdc25a deficiency is partially compensated by activity of cdc25d, the only other identified zebrafish cdc25, cdc25d has been characterized as a highly divergent isoform of cdc25. Zebrafish cdc25d functionally complements Schizosaccharomyces pombe cdc25 activity (19); however, its function in zebrafish has not been established. MO knockdown of cdc25d alone led to a mild growth defect (Fig. 3C) but knockdown of cdc25d in cdc25a/C0/C0 embryos led to a much more significant growth defect (Fig. 3D), as did double knockdown of cdc25a and cdc25d in wild-type embryos (Fig. 3E). These results indicated that cdc25d activity contributes to the early development of cdc25a/C0/C0 embryos. On the basis of these results, we asked whether overexpression of cdc25d might at least partially rescue the cell proliferation defect of cdc25a/C0/C0 embryos. To test this possibility, we injected cdc25a/C0/C0 embryos with cdc25d mRNA and stained for pH3 (Fig. 3F and G). Embryos injected with cdc25d mRNA had a slightly increased number of pH3 foci as compared with control cdc25a/C0/C0 embryos (the number of pH3-positive cells in a defined area of the tail was 19.3 ± 11.3 for cdc25a/C0/C0 embryos and 35.1 ± 13.1 for cdc25a/C0/C0 embryos injected with cdc25d mRNA; P = 0.059 by 2-tailed Student t test). cdc25d mRNA did not increase the number of pH3-positive cells in wild-type embryos (118.5 ± 22.3 pH3-positive cells in control vs. 131 ± 19.6 in cdc25d mRNA injected; P = 0.39). cdc25d mRNA injection did not rescue morphology or survival in cdc25a/C0/C0 embryos. Some caution is warranted in interpreting the cdc25d gain of function experiments, because they relied on overexpression and because the rescue was incomplete. However, taken together with the loss of function data, these results strongly suggest that cdc25 activity is necessary for early embryonic development in zebrafish, and that cdc25a and cdc25d are at least partially redundant.

**Inhibition of ATM allows G2–M transition in cdc25a mutants**

While overexpression of cdc25d increased the number of mitotic cells in cdc25a/C0/C0 mutants, endogenous levels of cdc25d activity were unable to rescue cdc25a deficiency, despite the fact that cdc25d is expressed during early development (19). It is possible that cdc25d activity is simply too weak to complement cdc25a deficiency. However, we also

Figure 3. Knockdown of cdc25d leads to synergistic growth defects in the absence of cdc25a. A, control MO-injected wild-type (wt) embryo showing normal morphology. B, control MO-injected cdc25a/C0/C0 embryo. Knockdown of cdc25d in wt embryos (C) causes mild morphologic defects, whereas knockdown of cdc25d severely impairs the growth of cdc25a/C0/C0 mutants (D). Similar severe growth defects result from simultaneous cdc25a and cdc25d knockdown in wt embryos (E). F, anti-pH3 staining of cdc25a/C0/C0 mutant. G, anti-pH3 staining of cdc25a/C0/C0 mutant injected with 25 ng/μL cdc25d mRNA.
considered the possibility that another factor inhibits cell proliferation in cdc25a mutants. We noted that the phenotype of cdc25a−/− mutant embryos (accumulation in G2 phase before the onset of chromatin condensation as signaled by pH3 positivity) is also compatible with cell-cycle arrest due to activation of the G2–M checkpoint. The G2–M checkpoint is dependent on the action of the PIKK family member ATM; it is present in zebrafish embryos and is robustly activated by DNA-damaging agents, such as gamma radiation (Supplementary Fig. S4).

To test whether the G2–M checkpoint is activated in cdc25a−/− embryos, we treated the embryos with the ATM inhibitor KU55933. To show that KU55933 inhibits zebrafish ATM, we pretreated wild-type embryos with 10 μmol/L KU55933 for 4 hours, then exposed pretreated embryos to 12 Gy ionizing radiation. At 48 hpf, irradiated KU55933-treated embryos displayed severe morphologic defects, similar to those present in irradiated embryos after MO knockdown of ATM (29; Supplementary Fig. S5).

Having established that KU55933 is active in fish, we next tested whether the G2–M accumulation in cdc25a−/− mutants was the result of activation of the G2–M checkpoint. We crossed heterozygous cdc25a+/− fish and treated the resulting embryos with KU55933 from 12 to 24 hpf, then fixed the embryos and stained for pH3 (Fig. 4E). The pH3 levels were quantified by counting the number of pH3 foci in a defined area of the embryo (Fig. 4G). Control DMSO-treated clutches displayed the expected 25% of embryos with a very low number of pH3-positive cells as compared with wild-type embryos (Fig. 4A, C, and G). In contrast, cdc25a−/− embryos treated with KU55933 exhibited a marked increase in the number of pH3-positive cells (Fig. 4D and G; P < 0.0001). We obtained identical results by inhibiting ATM with caffeine (Supplementary Fig. S6). We confirmed that the embryos with increased cell proliferation after KU55933 treatment were genotypically cdc25a−/− using a restriction fragment length polymorphism specific for the standstill cdc25a mutant allele (not shown).

To rule out an off-target effect of KU55933 and provide an independent means of inhibiting ATM, we injected 0.2 mmol/L control or ATM-specific MOs (29) into offspring of heterozygous cdc25a+/− fish, and assessed cell proliferation using pH3. Compared with control MO-injected embryos, ATM MO-injected embryos had increased cell proliferation (Fig. 4E and F). Further confirming these results, we found that cdc25a−/− embryos contain elevated levels of phospho-Y15-Cdk2, and that inhibition of ATM results in reduction of this inhibitory phosphorylation (Fig. 4H). We also considered the possibility that the phenotype of cdc25a−/− mutants is due at least in part to activation of a replication checkpoint due to replication stress. To test this possibility, we knocked down expression of ataxia-telangiectasia and rad9-related (arr), another PI3K family member that delays or arrests the cell cycle in response to stalled replication forks or other forms of replication stress (25). Knockdown of arr did not rescue the proliferation defect of cdc25a morphants (Fig. 4G). Furthermore, cdc25a morphants did not exhibit elevated levels of the phosphorylated form of CHK1 protein, as would be expected if the replication checkpoint were activated (Supplementary Fig. S7E).

To test whether the increased proliferation in KU55933-treated cdc25a−/− embryos was due to a true increase in cell-cycle progression or merely indicative of a release of the G2–M checkpoint into mitosis, we conducted fluorescence-activated cell sorting (FACS) analysis on cdc25a−/− embryos and phenotypically wild-type clutches treated either with the KU55933 or 1% DMSO (samples 1 and 2, Fig. 4J). While cdc25a−/− embryos treated with DMSO accumulated in G2 (sample 3), cdc25a−/− embryos treated with the ATM inhibitor (sample 4) exhibited a large G1 peak, indicating that they had resumed the cell cycle. Quantification of the FACS plots revealed the following distributions: cdc25a−/− in DMSO: 30.6% G1, 17.8% S, 64.6% G2; cdc25a−/− in KU55933: 61.5% G1, 31.2% S, and 12.8% G2. For comparison, the values for wild-type in DMSO are: 49.7% G1, 32.2% S, and 17.6% G2; and wild-type in KU55933: 49.7% G1, 36.1% S, and 15.8% G2. The large fraction of cells in G1 in KU55933-treated cdc25a−/− embryos likely reflects the release of a large number of G2 cells into mitosis and a subsequent G1.

These results indicated that G2–M accumulation in cdc25a−/− embryos was largely due to activation of the ATM-dependent G2–M checkpoint, and that cell proliferation in the mutant embryos could resume once ATM activity was inhibited. Having shown that cdc25a and cdc25d activities are at least partially redundant (Fig. 3), we hypothesized that in KU55933-treated, cdc25a-deficient embryos, cdc25d activity drives the G2–M transition. To test this model, we knocked down cdc25d in cdc25a−/− embryos and tested whether the cell cycle resumed after ATM inhibition. We found that, in the absence of expression of either cdc25a or cdc25d, KU55933 treatment failed to rescue the cell proliferation defect (Fig. 4G, 1, and K). Thus, in the absence of cdc25a activity, ATM seems to exert its regulatory effect on cdc25d.

cdc25 activity is also regulated at the G1–S checkpoint, principally by p53 and its downstream target p21 (8). To determine whether activation of the G1–S checkpoint contributed to the cell-cycle phenotype of cdc25a-deficient embryos, we knocked down cdc25a in p53214h mutant embryos, which are deficient in p53-mediated responses (35). We also simultaneously knocked down cdc25a and cdkn1a, which encodes p21. Neither p53 deficiency nor p21 deficiency increases the number of proliferating cells in cdc25a-deficient embryos (Supplementary Fig. S7A and S7B). Finally, we stained wild-type and cdc25a−/− embryos for the phosphorylated form of Rb protein, an indicator of G1–S cyclin-Cdk activity. Rb protein was phosphorylated in both wild-type cdc25a-deficient embryos, indicating that the G1–S transition was occurring in the mutants (Supplementary Fig. S7C and S7D).

ATM is activated during normal embryonic developmental cell cycles
Having established that an ATM-dependent G2–M checkpoint is present in cdc25a−/− embryos, we investigated
the mechanism of checkpoint activation. We first considered the possibility that Cdc25a deficiency results in increased DNA DSBs in developing embryos. We used the comet assay to measure the amount of DNA damage on a single-cell basis (36). We have recently adapted this method to measure DNA damage in zebrafish cells (24). Conducting the comet assay on 24 hpf cdc25a−/− embryos, wild-type embryos, or wild-type embryos treated with 12 Gy of ionizing radiation as a positive control, we observed a slight decrease in spontaneous DNA damage in the cdc25a−/− mutants (Fig. 5A–D). While it is possible that the comet assay fails to detect minor amounts of DNA damage, these data indicate that loss of cdc25a itself does not induce severe DNA damage. The observed decrease in comet tail size in cdc25a−/− mutants may be due to decreased spontaneous replication-related DNA damage due to G2–M cell-cycle arrest.

Figure 4. Inhibition of ATM attenuates the cell proliferation defect in cdc25a−/− embryos. Twenty-four hour wild-type (wt; A and B) and cdc25a−/− (C and D) embryos treated from 12 to 24 hpf with 1% DMSO (A and C) or 10 μmol/L KU55933 ATM kinase inhibitor (B and D) and stained for pH3. E and F, genetic knockdown of ATM in cdc25a−/− embryos. Injection of cdc25a−/− embryos with control MO (E) has little effect, whereas injection of an ATM MO (F) leads to increased cell proliferation. G, quantification of the number of pH3 foci present in the tails of embryos under different conditions. †, P < 0.01; ‡, P < 0.001; ns: not significant). Inhibition of ATM significantly increases the number of proliferating cells in wt and cdc25a-deficient embryos. Knockdown of atr slightly increases pH3 number in wt but not cdc25a-deficient embryos. H, immunoblot analysis of total Cdc2 and phospho-tyrosine 15 Cdc2 in wt and cdc25a−/− embryos treated with DMSO and KU55933. Inhibition of ATM increases cdc25 phosphatase activity and reduces pCdc2 in cdc25a−/− embryos. I, DNA content FACS profiles of wt embryos treated with DMSO (sample 1) or KU55933 (sample 2) and cdc25a−/− embryos treated with DMSO (sample 3) or the ATM inhibitor (sample 4). Inhibition of ATM in cdc25a−/− embryos rescues progression of the cell cycle, indicated by a larger G1 peak, as compared with cdc25a−/− treated with DMSO. J and K, inhibition of ATM activity in zebrafish does not restore cell proliferation in embryos lacking both cdc25a and cdc25d. Embryos injected with MOs targeting both cdc25a and cdc25d transcripts treated with DMSO (J) or 10 μmol/L KU55933 (K) exhibit no change in mitotic cells (quantified in G).
H2AX (pH2AX) present in cells of unirradiated wt embryos, type and DSBs (37). We conducted immunoblot analysis on wild-

designated zebrafish rabbit polyclonal antibody speci-

gene

embryos do not exhibit increased DNA damage, as measured

– DMSO (lanes 1

– mutant embryos, or 12 Gy irradiated (IR) wt embryos pretreated with (8)

cdc25a

– DMSO (lanes 1–3) or KU55933 (4–6). pH3 is induced in wt by IR, and this effect is blocked by KU55933. However, cdc25a−/− embryos do not exhibit increased levels of γ-H2AX.

As an alternative approach to detect DNA damage foci in zebrafish, we developed an anti–phospho-histone H2AX rabbit polyclonal antibody specific for phosphorylated zebrafish histone H2AX (23). Phosphorylated histone H2AX, designated γ-H2AX, is present at histone cores flanking DSBs (37). We conducted immunoblot analysis on wild-type and cdc25a−/− embryos to measure γ-H2AX levels (Fig. 5E). Compared with wild-type controls, cdc25a−/− embryos have similar to decreased γ-H2AX levels. As expected, irradiated wild-type embryos exhibit increased γ-H2AX, which can be reduced by pretreatment of the embryos with KU55933. Therefore, the ATM–γ-H2AX axis is present and normally functioning in zebrafish, but cdc25a−/− embryos do not exhibit increased DNA damage, as measured by histone H2AX phosphorylation. The low level of γ-H2AX in cdc25a−/− embryos made it unlikely that widespread DNA damage could account for the observed profound cell-cycle arrest. As a further evidence of this point, we tested whether H2AX phosphorylation precedes the onset of the cell-cycle phenotype in cdc25a−/−, by staining age-
matched embryos for γ-H2AX and pH3 (Supplementary

– M accumulation in developing embryos, but the accumulation is not attributable to increased DNA DSBs. To account for ATM activity in cdc25a−/− embryos in the absence of detectable DNA damage, we next considered the possibility that Cdc25A directly regulates ATM via Cdc25's phosphatase activity. To test the possibility that Cdc25A directly dephosphorylates and regulates ATM, we overexpressed mouse Cdc25A in A549 cells for 48 hours, irradiated the cells, and assessed the phosphorylation status of ATM and its target, CHK2, by immunoblotting (Supplementary Fig. S9). Both ATM and CHK2 showed increased phosphorylation status after irra-
diation, and overexpression of Cdc25A did not decrease the phosphorylation, indicating that Cdc25A does not directly regulate ATM. We also noted increased phospho-ATM in nonirradiated Cdc25A-overexpressing cells consistent with previous findings that overexpression of Cdc25A leads to increased DNA damage (38).

Finally, we investigated whether ATM is generally active during early developmental cell cycles, and if its effects on the cell cycle were being unmasked by cdc25a deficiency in the mutant embryos. Indeed, wild-type embryos treated with KU55933 and quantified for the number of mitotic cells using pH3 as a marker exhibited an increased number of mitotic cells as compared with controls (Fig. 4A, B, and G); however, this effect did not reach statistical significance (P = 0.09). These results suggest that, during normal develop-
ment, ATM is active and may attenuate the percentage of cells progressing from G2 to M phase. On the basis of the above results, we conclude that ATM is constitutively active during development but does not completely deplete Cdc25 activity. Thus, in wild-type animals, a balance between cell-
cycle arrest/delay and cell-cycle progression is maintained.

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an earlier report of lower survival in ATM morphants (29) and suggests ATM may play an important role in normal development, even in the absence of exogenous genotoxic stress.

**Discussion**

ATM was first identified as the protein mutated in the clinical syndrome ataxia-telangiectasia, which is characterized by immune dysfunction, abnormal sensitivity to ionizing radiation, cerebellar ataxia, and telangiectases. Subsequent work revealed that ATM, and the related PIKKs ATR and DNA-PK, are central regulators of the DNA damage response (39, 40). In response to genotoxic stresses causing DNA DSBs, one action of ATM is to mediate G2–M cell-cycle arrest by acting through the checkpoint kinases CHK1/CHK2 to target Cdc25 for destruction (41–43). In keeping with this role of ATM in maintaining genomic integrity, people with mutations in the ATM gene are at increased risk of developing cancer (44, 45).

More recently, it has become clear that the PIKKs and their downstream partners have important roles in maintaining normal cell homeostasis, even in the absence of exogenous sources of DNA damage. ATM and ATR control the timing of replication origin firing, in the absence of DNA damage (46). CHK1 localized to the centrosome has been shown to regulate Cdc25B activity to control the entry into mitosis (47). Constitutive histone H2AX phosphorylation and constitutive ATM activation (CAA) in normal cycling cells has been postulated to occur and has been attributed in interphase cells to DNA damage due to endogenous oxidative stress (48–50). In mitotic cells, CAA and H2AX phosphorylation have been associated with chromatin condensation, and postulated to play a role in maintaining mitotic spindle integrity (51, 52).

Here, we show that a G2–M accumulation is present in developing cdc25a mutant embryos, as evidenced by loss of pH3 positive nuclei and by DNA content profiling. This accumulation is due to activation of ATM and can be partially overcome by inhibiting ATM, so long as cdc25d activity is present. The G2–M accumulation is not due to increased DNA damage, but seems to be constitutive, as inhibiting ATM in wild-type embryos also increases the number cells making the G2–M transition. In the cdc25a<sup>−/−</sup> mutants, we do not believe that ATM activation results from premature chromatin condensation or other mitotic abnormalities, because cells in developing mutant embryos arrest before the onset of chromatin condensation as evidenced by markedly reduced levels of histone H3 serine 10 phosphorylation.

We hypothesize that a basal level of ATM activity occurs in normal cell cycles. In cdc25a<sup>−/−</sup> cells, early embryonic development proceeds fairly normally due to compensatory phosphatase activity of cdc25d. In cells with normal levels of cdc25 activity, the basal level of ATM activity subtly attenuates cell-cycle progression; however, in cdc25a<sup>−/−</sup> embryos, the phosphatase activity of cdc25d is overwhelmed by basal ATM activity, leading to cell-cycle accumulation in the absence of marked DNA damage (Fig. 6). Only when ATM

![Figure 6. Model of hierarchical Cdc25 function during normal cell cycles and response to stress. In the model, the size of the font indicates the relative abundance of a protein or isoform. A, during a normal cell cycle, ATM is constitutively active but at a low level. The degree of inhibition of Cdc25a and Cdc25d by ATM/CHK1 is insufficient to prevent Cdc25 phosphatase activity. Therefore, inactive cdc2-P is converted to active cdc2* , fostering the G2–M transition. B, in the presence of DNA DSBs, ATM is strongly activated, leading to downregulation of cdc25 activity. Cdc2 remains in the inactive, phosphorylated state, and G2–M progression is inhibited. C, when cdc25a is deficient, cdc25d is present but may be inhibited by ATM. Residual cdc25d activity is too low to fully convert inactive cdc2-P to active cdc2* , resulting in delay of the cell cycle and accumulation in G2. D, inhibition of ATM in cdc25a-deficient cells unmasks the activity of cdc25d. Under these conditions, sufficient cdc2-P is converted to active cdc2*, and G2–M progression is maintained.](mcr.aacrjournals.org)
activity is genetically or pharmacologically abrogated can cell-cycle activity resume. Thus, we propose that a hierarchy of Cdc25 activity is present, such that enough combined Cdc25 phosphatase activity must be present to overcome the basal ATM activity to progress the cell from G2 to M phase. Our data do not indicate the mechanism(s) regulating Cdc25. The sequence of Cdc25 is poorly conserved relative to other Cdc25 family members (19), and we did not identify conserved consensus phosphorylation sites.

Because of our observation that G1–M accumulation is likely due to basal ATM activity, we sought the mechanism by which Cdc2 is being dephosphorylated in ATM-inhibited embryos. We discovered that zebrafish Cdc25d is expressed and is able to mediate cell-cycle progression to a modest degree. Knockdown of Cdc25d had profound consequences for development in cdc25a^{-/-} or cdc25a knockout embryos, the first demonstration that Cdc25d is active in vivo in zebrafish. Ectopic expression of Cdc25d led to modest increases in the number of proliferating cells that was detectable in cdc25a^{-/-} embryos. Previous reports indicated that Cdc25 could rescue a yeast cdc2 mutant but failed to show activity in zebrafish (19,20). Consistent with these results, we did not detect a significant effect in wild-type embryos, suggesting that Cdc25a is responsible for the majority of Cdc25 activity during development. Expression of Cdc25d did not, however, rescue the morphologic phenotype of cdc25a^{-/-} embryos. This finding may be due to limited ability of Cdc25d to oppose ATM activity in cdc25a^{-/-} mutants, or may point to essential roles of Cdc25a in processes other than G1–M progression.

The early development of vertebrate embryos requires rapid and highly regulated cell divisions. Cdc25 phosphatases are important mediators of this rapid cell-cycle flux. Balancing this rapid growth is the need to respond to genotoxic insults from exogenous or endogenous agents to ensure the health and genomic stability of the developing embryo. The results presented here emphasize the delicate balance between cell proliferation and cell-cycle arrest/delay, and highlight the competing roles of ATM and Cdc25 family members in this process. The cdc25a^{-/-} mutant also provides a sensitive model of physiologic ATM activity that could be used to further dissect the genetics of the DNA damage response and to screen for novel checkpoint inhibitors.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References


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