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The genomic repertoire for cell cycle control and DNA metabolism in *S. purpuratus*

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Abstract

A search of the *Strongylocentrotus purpuratus* genome for genes associated with cell cycle control and DNA metabolism shows that the known repertoire of these genes is conserved in the sea urchin, although with fewer family members represented than in vertebrates, and with some cases of echinoderm-specific gene diversifications. For example, while homologues of the known cyclins are mostly encoded by single genes in *S. purpuratus* (unlike vertebrates, which have multiple isoforms), there are additional genes encoding novel cyclins of the B and K/L types. Almost all known cyclin-dependent kinases (CDKs) or CDK-like proteins have an orthologue in *S. purpuratus*; CDK3 is one exception, whereas CDK4 and 6 are represented by a single homologue, referred to as CDK4. While the complexity of the two families of mitotic kinases, Polo and Aurora, is close to that found in the nematode, the diversity of the NIMA-related kinases (NEK proteins) approaches that of vertebrates. Among the nine NEK proteins found in *S. purpuratus*, eight could be assigned orthologues in vertebrates, whereas the ninth is unique to sea urchins. Most known DNA replication, DNA repair and mitotic checkpoint genes are also present, as are homologues of the pRB (two) and p53 (one) tumor suppressors. Interestingly, the p21/p27 family of CDK inhibitors is represented by one homologue, whereas the INK4 and ARF families of tumor suppressors appear to be absent, suggesting that these evolved only in vertebrates. Our results suggest that, while the cell cycle control mechanisms known from other animals are generally conserved in sea urchin, parts of the machinery have diversified within the echinoderm lineage. The set of genes uncovered in this analysis of the *S. purpuratus* genome should enhance future research on cell cycle control and developmental regulation in this model.

Keywords: Sea urchin; Cell cycle; Replication; Mitosis; Checkpoint; Kinases

Introduction

The eukaryotic cell cycle orchestrates cell growth, the replication and segregation of genomic DNA, and cell division. While its core mechanisms are highly conserved, with the evolution of animals, the cell cycle became contingent on intercellular mitogenic signaling and thereby subjugated to the variety of ontogenetic programs found in different animal clades.

Progression through the eukaryotic cell cycle is controlled by a complex network of kinase activities. Central to this

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machinery is the family of cyclin-dependent kinases (CDKs), which phosphorylate a set of regulatory factors. The activity of these serine/threonine kinases is in turn controlled at multiple levels: by synthesis of the regulatory cyclin subunits, by phosphorylation of both the catalytic and cyclin subunits, by binding to CDK inhibitors (CKIs), by subcellular localization, and by specific ubiquitylation targeting of cyclins and CKIs to proteasome-mediated proteolysis (Obaya and Sedivy, 2002). Cyclins involved in cell cycle control have been classified as G1-, S-, or M-phase regulators, based on when their activities are required in the cell cycle (Pines, 1999). Cyclin D-dependent kinases CDK4/CDK6 collaborate with cyclin E/CDK2 to promote phosphorylation of the retinoblastoma protein (pRB) and its relatives p107 and p130, relieving the inhibition of E2F family transcription factors and thus stimulating progression from G1 to S phase. The G1 phase of the cell cycle is characterized by low CDK activity and replication origins being assembled in a pre-replicative state competent to undergo DNA replication in the subsequent S phase. Assembly of pre-replicative complexes (pre-RC) is marked by the sequential binding of ORC (for "origin recognition complex"), Cdc6, Cdt1, and finally the DNA helicase MCM2–7 (for "mini chromosome maintenance") complex to the origin of replication (reviewed in Bell, 2002; Spradling, 1999). Activation of CDK2 and of the heterodimeric kinase Cdc7/Dbf4 at the G1–S transition drives the cell into a state where replication can be initiated but new pre-RC cannot be assembled, ensuring that chromosomal DNA replicates only once per cell cycle (reviewed in Blow and Dutta, 2005; Woo and Poon, 2003). Completion of S phase triggers the activation of mitotic kinases which fully activate cyclin B/CDK1, initiating the complex program of chromosome segregation. Exit from mitosis is marked by the shutdown of CDK activity that follows the rapid degradation of cyclin B. Cyclin B, with other critical substrates for cell cycle progression, is targeted for degradation by the ubiquitin-dependent pathway.

The core genes involved in these biological processes have been found in animals, plants, and unicellular organisms such as yeast. However, some regulatory mechanisms have evolved differently. For example, multiple gene isotypes (e.g. those encoding cyclins) are present in certain lineages, the precise number varying between different organisms. Here we surveyed the genome of *Strongylocentrotus purpuratus* to obtain an insight into how echinoderms either conserve or have modified these fundamental systems.

The sea urchin has been an important model for many years in the study of basic biology. Crucial in developmental biology, it has also been essential in cell cycle studies, a key example being the identification of the first cyclin. It was in *Arbacia punctulata* that Tim Hunt first noticed the "unexpected behavior" of a protein synthesized early after fertilization that almost disappeared before cleavage and was "henceforth called cyclin" (Evans et al., 1983). Together with studies on amphibians, data obtained in marine invertebrates, i.e. the detection in starfish oocytes of a cytoplasmic activity which mediates maturation-inducing hormonal action and brings about germinal vesicle breakdown (Kishimoto and Kanatani, 1976), the demonstration of the universality of this M-phase promoting factor (MPF) (Kishimoto et al., 1982), the sequencing of Clam cyclin A (Swenson et al., 1986), and the purification of cyclin B/CDK1 from starfish (Labbe et al., 1989) were decisive for understanding the mechanism underlying meiotic and mitotic onset (MPF reviewed in Kishimoto, 1999; Masui, 2001).

A key feature of sea urchins is that their gametes are stored as haploid eggs. Thus, fertilization directly triggers entry into mitosis rather than resumption of meiosis as in most animal eggs. After one cycle in which short G1 and G2 phases precede DNA replication and mitosis respectively, a rapid succession of S and M phases marks the cleavage stages. These early divisions rely on translation of maternally stored mRNA or post translational modifications of existing proteins stored in the eggs until the cell cycle becomes dependent on zygotic transcription and develops into a more conventional somatic cycle with gap phases.

The sea urchin occupies an important evolutionary position with respect to vertebrates because the echinoderms and their sister group hemichordates are the only other deuterostome animals beside the chordates. The sea urchin is thus more closely related to humans than are the other major invertebrate models *Drosophila* and *C. elegans*. The sequenced genome of *S. purpuratus* strengthens the sea urchin as a model organism for studying the regulatory networks that control cell proliferation, with potentially important implications for understanding their human counterparts.

In this paper, we describe the sea urchin repertoire for cell cycle control and DNA metabolism as identified in the genome sequence of *S. purpuratus* and supplement this description with currently available functional data.

Materials and methods

Identification of cell-cycle- and DNA-replication-related genes

To identify cell-cycle-related genes in the *S. purpuratus* genome, BLAST searches were performed against the Baylor College of Medicine (BCM) sea urchin database. Sequences that lack a cyclin domain according to Pfam domain analysis were eliminated. Computationally predicted models of other cell cycle genes were collected by searching the GLEAN3 gene set at Baylor, either by using BLASTP homologous amino acid sequences from known cell cycle control genes or by using the PFAM search engine set up on the Baylor annotation site. Domains were analyzed through the SMART (simple modular architectural research tool) web interface from http://smart.embl-heidelberg.de/ or the ScanProsite search engine from http://www.expasy.ch/. Nek Alignment was done using ClustalW at http://www.ebi.ac.uk/clustalw/.

For phylogenetic trees, protein sequences were aligned with MAFFT (Katoh et al., 2005, 2002) using E-INS-i strategy. The conserved domains were selected and manually improved using Seaview (Galtier et al., 1996). In order to make a Maximum Likelihood analysis, Prottest (Abascal et al., 2005) was used to determine the best amino acid substitution matrix, RtREV+I+G+F was the most suitable for our data. This matrix was used to run the local version of PHYML (Guindon et al., 2005) to infer the ML tree. Trees were validated with a bootstrap procedure using 1000 replicates. Treeillustrator (Trooskens et al., 2005) was used to draw the final tree.

Identification of DNA repair genes

The Gene Ontology database at Mouse Genome Informatics (MGI) (http://www.informatics.jax.org/) was searched with the term "DNA repair". Under DNA repair ([GO:0006281]; {Biological process{DNA metabolism{DNA

repair}}}), the subgroups base excision repair [GO:0006284] (17 genes, 20 annotations), double-strand break repair [GO:0006302] (8 genes, 10 annotations), nucleotide excision repair [GO:0006289] (19 genes, 21 annotations) and mismatch repair [GO:0006298] (7 genes, 8 annotations) were followed to retrieve all of the individual MGI entries. The peptide sequence for each retrieved entry was used to query the *S. purpuratus* GLEAN3 peptide sequence predictions through the BLAST server at Baylor. The results of that search are displayed in Table S2. Some mouse genes with repair annotations had no clear homolog in the GLEAN3 set and in that case the assembled *S. purpuratus* genome sequence and available ESTs were directly searched to confirm the absence. Xrcc1, not present as a base excision repair factor at MGI, was added to the base excision repair list.

Results and discussion

The kinase network

The cell cycle clock is operated by a network of kinase activities, at the core of which are the cyclin and CDK families. The regulatory cyclin subunits not only activate CDKs by changing the conformation at their catalytic sites, but also contribute to the selection of CDK substrates, subcellular localization, and regulation of protein stability (reviewed in Malumbres and Barbacid, 2005; Murray, 2004; Sanchez and Dynlacht, 2005; Sherr and Roberts, 2004). On top of this core machinery, additional kinases, often monomeric, regulate various events during the cell cycle. These include the mitotic kinases, aurora, polo, the NimA-related kinases (NEK), or the checkpoint kinases Chk (reviewed in Nigg, 2001). The mitogen-activated protein kinases (MAPK) signaling cascades which regulate meiotic and mitotic cycles are described in an accompanying paper (Bradham et al., 2006).

Cyclin/CDK complexes

Cyclins were among the first characterized cell cycle regulators (Evans et al., 1983) and since their identification a large number of cyclin genes have been identified in various organisms. On the basis of sequence similarity, expression pattern, and protein activity during the cell cycle, cyclins have been grouped into several classes. In animals, at least 13 classes (A to L and T) of cyclins have been described (Pines, 1995), and the human genome encodes a total of 22 cyclins. Not all cyclins in animals are demonstrated to be involved in cell cycle regulation, and only cyclins A, B, D, and E are commonly considered to be part of the core cell cycle control machinery. A second set of cyclins, cyclins K, L and T, are involved in transcription or mRNA processing (Loyer et al., 2005), while a third set, cyclin C and H, have been implicated in regulation of both cell cycle and gene expression. The functions of the remaining cyclins, cyclin F, G, I, and J, remain somewhat unclear.

All the distinctive classes of cyclins were found in the *S. purpuratus* genome (Fig. 1), which contains at least 16 genes encoding proteins with a cyclin domain. However, in contrast to the human genome, which includes various isotypes of several of these cyclins, the sea urchin genome contains a single gene for each defined subfamily, with the exception of cyclins of the B and K/L types.

The *S. purpuratus* genome sequence confirmed that the previously identified G1–S cyclins, cyclin D and E (Moore et al., 2002; Sumerel et al., 2001), are indeed the only members of these respective subfamilies in sea urchins. Cyclin D mRNA is barely detectable in eggs and early embryos but increases dramatically at the early blastula stage, remaining thereafter at a constant level throughout embryogenesis (Moore et al., 2002). Premature expression of cyclin D in cleavage embryos is lethal; however, while cyclin D expression is required for normal development, it may not be necessary for cell cycle control. Cyclin E is synthesized during oogenesis, and the level of cyclin E protein is maintained constant throughout early sea urchin embryogenesis. This implies that fluctuations in cyclin E are not required for cell cycle progression through the early embryonic cell cycles. Instead, it has been postulated that cyclin E may play a role in the remodeling of the sperm chromatin, a prerequisite for subsequent DNA replication (Schnackenberg and Marzluff, 2002). After the blastula stage, cyclin E mRNA and protein levels are very low, and cyclin E expression is predominant only in cells that are actively dividing, suggesting that cyclin E contributes to cell cycle control at these stages.

Mitotic cyclins include three distinct gene families, cyclins A, B, and B3 (Jacobs et al., 1998), that appear to have overlapping functional properties. As in other invertebrates including starfish (Table S1), surf clam, limpet, and fly (Lehner and O'Farrell, 1989; Okano-Uchida et al., 1998), a single cyclin A has been found in sea urchin. Cyclin A is present in sea urchin oocytes, but its synthesis is not required for meiotic maturation (Voronina et al., 2003). Fertilization increases synthesis of cyclin A which associates with CDK1 and CDK2 (Moreau et al., 1998). The function of cyclin A during S phase has not yet been demonstrated in sea urchin, but with cyclin B, it drives the G2–M transition by potentiating MPF activation during early embryogenesis (Geneviève A.M. et Picard A., unpublished data), as is the case in *Xenopus* (Devault et al., 1992) or starfish (Okano-Uchida et al., 2003). Moreover, cyclin A can compensate for an absence of cyclin B in early sea urchin embryos, demonstrating a functional overlap between the two mitotic cyclins (Voronina et al., 2003). Three B type cyclins were identified in *S. purpuratus*: the previously characterized sea urchin cyclin B (Lozano et al., 1998; Thatcher et al., 1995; Voronina et al., 2003), an orthologue of cyclin B3 (SPU_15285), and a novel cyclin B related to mitotic cyclins of plants (SPU_20986). A whole-genome analysis in *Caenorhabditis elegans* also revealed two novel B type cyclins that are closely related to the previously known cyclin B (Nieduszynski et al., 2002). A phylogenetic tree of B type cyclins (Fig. 2) shows that the novel *S. purpuratus* cyclin B is not related to any other cyclin B subfamily, a feature shared with a previously classified cyclin A-like protein in *Ciona intestinalis* (Kawashima et al., 2003), which is in fact more closely related to the sea urchin novel cyclin B than to A type cyclins (Figs. 1 and 2). These data reinforce evidence for multiple cyclin B gene duplication events (Nieduszynski et al., 2002). An early duplication event in animal evolution may have given rise to the B and B3 type cyclins, identified in all animals to date. More recent duplication events

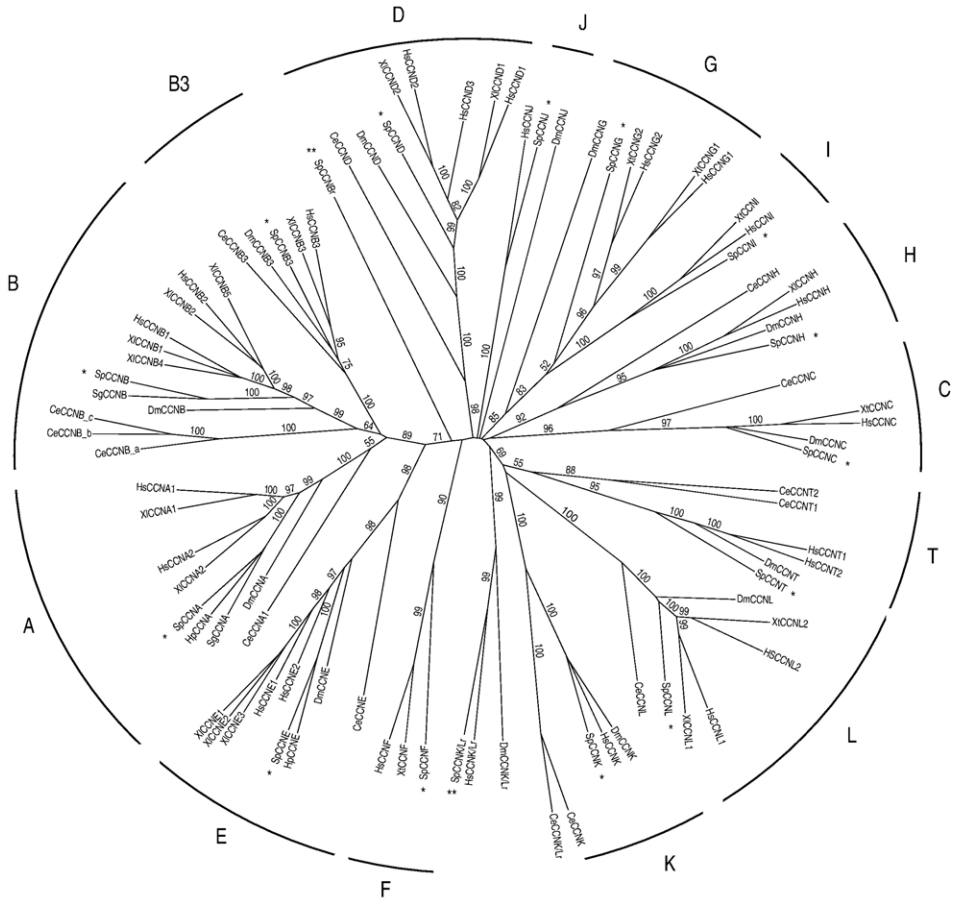

Fig. 1. Phylogenetic tree of cyclins, reconstructed by the Maximum Likelihood method (ML), with bootstrap values higher than 50% shown for each clade. Sequence Ids for the human (Hs), *Xenopus* sp. (Xl and Xt), *Drosophila melanogaster* (Dm), or *Caenorhabditis elegans* (Ce) cyclins (here noted CCN) are reported in Table S3. The *S. purpuratus* cyclins are noted with a star. The novel cyclins identified in *S. purpuratus* (Sp) which are not associated with an established cyclin subgroup are highlighted by two stars.

account for the variation in number of B type cyclins between organisms.

Cyclin C is one cyclin whose protein level does not vary significantly during the cell cycle or following growth factor stimulation. Recent analysis demonstrated that cyclin C, associated with CDK3, may play a role in the exit from quiescence, prior to pRb phosphorylation by CDK4/6/cyclin D1 in mammalian cells (Ren and Rollins, 2004). However, Cdk3 is mutated and thus non-functional in most laboratory strains of mice (Ye et al., 2001). While a potential cyclin C is present in the sea urchin, there is no apparent orthologue of the partner kinase, CDK3 (Table 1). Thus, the proposed mammalian function of cyclin C in the G0–G1 transition may not be conserved in all phyla. Cyclin C also associates with CDK8 (Leclerc and Leopold, 1996). An orthologue of CDK8 was identified in *S. purpuratus* (discussed below), which may be the only partner of cyclin C in the sea urchin. A second cyclin with a potential role both in cell cycle and transcription, cyclin H, is also present in the sea urchin genome. In vertebrates, cyclin H controls the activity of CDK7 as a Cdk activating kinase as well as its ability to phosphorylate the C-terminal domain of RNA-polymerase II (reviewed in Fisher, 2005; Harper and Elledge, 1998).

Cyclin K, T, and L constitute a protein subfamily with high sequence conservation within the conserved cyclin box and are members of the “transcription/mRNA processing” cyclin family (Loyer et al., 2005). The three cyclins have single potential orthologues in the sea urchin genome as displayed in Fig. 1. In addition, another gene of the cyclin K/L sub-type was identified in sea urchins (SPU_023343). This gene has potential orthologues in humans (XP_291577), mice (AAH07232), nematodes (NP_506615), and *Drosophila* (CG31232), the encoded proteins being uncharacterized to date. This suggests that new functions of cyclin proteins remain to be discovered.

Potential orthologues of cyclin F, G, I, and J were also found in the sea urchin genome. The expression of cyclin F in sea urchin embryo, as indicated by transcriptome tiling array data (Samanta et al., 2006), is in accordance with its proposed requirement for embryonic development (Tetzlaff et al., 2004).

Since their initial discovery in yeast, the CDKs have proven to be universal regulators of the cell cycle in all eukaryotes. It is generally admitted that cell cycle progression requires a different CDK/cyclin complex for each stage of the cell cycle. Thus, as mentioned in Introduction, progression through G1 is primarily under the control of CDK4/6 family members in association with cyclin D; CDK2, paired with

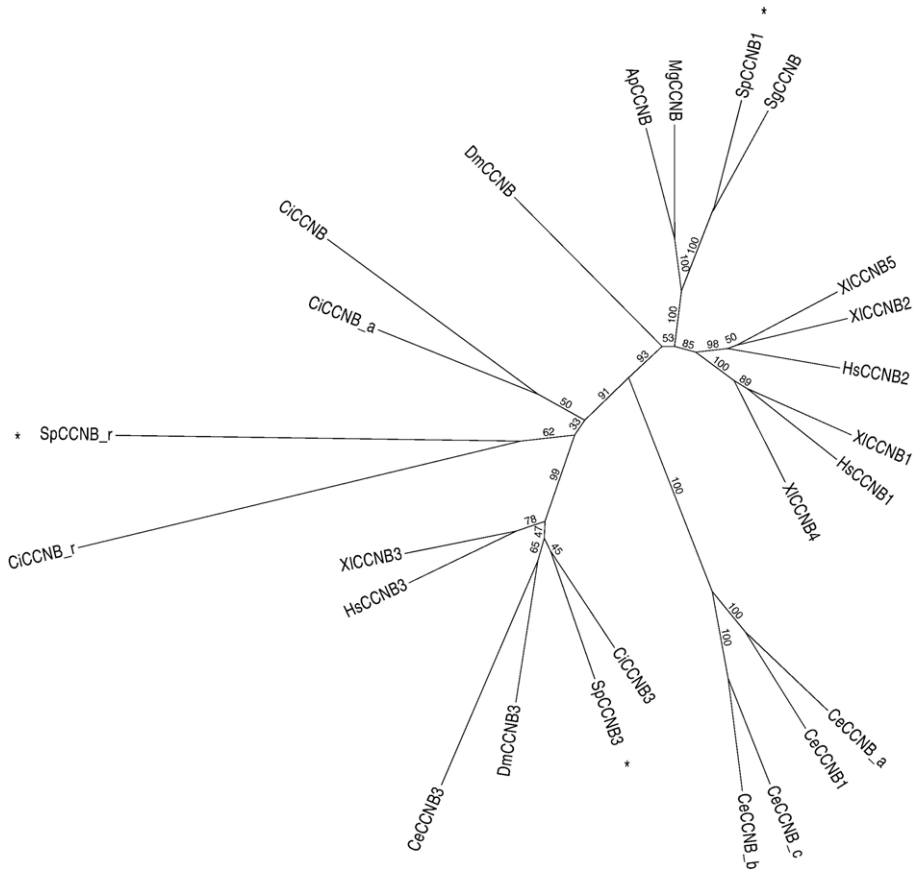

Fig. 2. Phylogenetic tree for selected B type cyclins. The tree was constructed by the Maximum Likelihood method (ML), with bootstrap values shown for each clade. The *S. purpuratus* cyclin B are noted with a star. Sequence Ids for the human (Hs), *Asterina pectinifera* (Ap), *Marthasterias glacialis* (Mg), *Xenopus laevis* (Xl), *Drosophila melanogaster* (Dm), *Caenorhabditis elegans* (Ce), and *Ciona intestinalis* (Ci) cyclin B (here noted CCNB) are reported in Table S3.

cyclin E and A, is required for G1–S transition and progression through S phase; while CDK1 together with cyclin A and B governs the G2–M transition. A second CDK network is critical for the regulation of transcription and mRNA processing (reviewed in Loyer et al., 2005). A few CDKs, such as CDK7, are involved in both regulatory networks. The sea urchin genome encodes most of the cell-cycle- or transcription-related CDKs encountered in mammalian cells, as displayed in Table 1. One exception is Cdk3, the absence of which was discussed above.

The core cell cycle genes Cdk1 and Cdk2 were previously identified in sea urchins (Moreau et al., 1998). Both kinases are constitutively expressed during early development. While the cyclin A and B/CDK1 activities peak in mitosis (Geneviere-Garrigues et al., 1995), CDK2 kinase activity is stable during early embryogenesis. In contrast to its role in somatic cells, CDK2 is dispensable for the activation of the first DNA replication after fertilization; however, CDK activities are required to maintain replication to one per cell cycle (Moreau et al., 1998).

Only one member of the CDK4/6 family was identified in the *S. purpuratus* genome. Similarly, a single homologue of Cdk4/6 is found in *D. melanogaster*, *C. elegans*, and *C. intestinalis*, supporting the view that the gene duplication is specific to vertebrates. Recent data from *D. melanogaster*, *C. elegans*, *S. purpuratus*, and mice suggest that cyclin D/CDK4 complexes are not essential for cell proliferation or early development in these species and may be involved more in the regulation of cell growth and/or developmental patterning than in cell cycle progression (Datar et al., 2000; Malumbres et al., 2004; Meyer et al., 2000; Moore et al., 2002; Park and Krause, 1999).

The network of CDK/cyclin complexes implicated in transcription and mRNA processing is conserved in *S. purpuratus*. Mammalian CDK7/cyclin H and CDK8/cyclin C, which phosphorylate the C-terminal domain (CTD) of RNA-polymerase II (RNAP II), regulate in an opposite manner the initiation of transcription, belonging respectively to the TFIIH and mediator complexes (reviewed in Loyer et al., 2005). CDK9, the CTD kinase subunit of P-TEFb, associated with cyclins T or K, positively controls the elongation phase of RNAP II transcription, while CDK11 bound to cyclin L has been implicated in transcription and pre-mRNA splicing. The latest kinase in this large family to be identified, CDK12 (previously CrkRS) and its close relative CDC2L5, are the highest molecular mass CDKs identified to date (Chen et al., 2006; Ko et al., 2001; Marques et al., 2000). Recently, both proteins have been shown to regulate pre-mRNA splicing (Chen et al., 2006; Even et al., 2006). A full-length CDC2L5 cDNA was initially identified in the sea urchin *Sphaerechinus granularis* (Marques et al., 2000). In this species, the mRNA is ubiquitously expressed throughout development, and in

Table 1
S. purpuratus cell cycle genes identified in this study

Biochemical process/function	Family	Spu Id	Assigned name	Role of homologues	Embryonic expression
Ser/Thr kinase	CDK	002210	Sp-CDK1	G2–M	+
		007655	Sp-CDK2	G1–S	+
		Not found	Sp-CDK3		
		000151	Sp-CDK4/6	G1–S	+
		017705	Sp-CDK5	Differentiation	+
		027370	Sp-CDK7	Cell cycle/transcription	− [a]
		001690	Sp-CDK8	Transcription	+
		012674	Sp-CDK9	Transcription	+
		006069/026319	Sp-CDK10		+
		016748/017385	Sp-CDK11	Cell cycle/mRNA processing	+
		028236	Sp-CDK12/CDC2L5	mRNA processing	+
		016385	Sp-PCTAIRE		+
		003654	Sp-PFTAIRE		−
CDK regulatory subunit	Cyclin	003528	Sp-cyclin A	S–G2–M	+
		015285/026576	Sp-cyclin B	G2–M	+
		006444	Sp-cyclin B3	G2–M	+
		003967	Sp-cyclin C	Cell cycle/transcription	−
		007013	Sp-cyclin D	G1–S	+
		001712	Sp-cyclin E	G1–S	+
		006781/007933	Sp-cyclin F		+
		009985	Sp-cyclin G		−
		011658	Sp-cyclin H	Cell cycle/transcription	−
		007312	Sp-cyclin I		+
		021704	Sp-cyclin J		−
		005021/018199	Sp-cyclin K	Transcription	+
		014989/011295/000328	Sp-cyclin L	mRNA processing	+
		021812	Sp-cyclin T	Transcription	+
		023343	Sp-cyclin K/Lr		+
		020986	Sp-cyclin Br		+
Ser/Thr kinase	CAK	004789	Sp-Ccrk		+
Tyr kinase	Wee1	014185/027012	Sp-Wee1	S–G2–M	+
	Myt1	008280	Sp-Myt1	S–G2–M	+
Tyr phosphatase	CDC25	019568	Sp-CDC25	S–G2–M	+
Mitotic kinase	PLK	017949	Sp-PLK1	G2–M	+
		000468	Sp-PLK2	G2–M	+
		016352	Sp-PLK4	G2–M	+
	Aurora	027833/027666	Sp-Aurora	G2–M	+
	NEK	019021	Sp-Nek1		+
		005277	Sp-Nek2	Mitosis	+
		023369	Sp-Nek4		+
		028348	Sp-Nek6/7		+
		005411	Sp-Nek8		+
		007456	Sp-Nek9		+
		018375/023408	Sp-Nek10		+
		014659/025176	Sp-Nek11		+
		017790	Sp-Neka		+
Checkpoint kinase	ATM	011072/025176	Sp-ATM	DNA damage/replication	−
	ATR	011017/026783	Sp-ATR	DNA damage/replication	+
	Chk1	007530	Sp-Chk1	DNA damage/replication	+
	Chk2	004975	Sp-Chk2	DNA damage/replication	+
CDK/Cyclin inhibitor	Cip/Kip	005856	Sp-Ckip1	G1–S–G2–M	+
CDK/Cyclin regulator	Mat1	014250	Sp-Mat1		+
Transcription factor/cofactor	Myb	000861	Sp-Myb	G1–S	+
	Myc	003166	Sp-Myc	G1–S	+
	E2F/DP	006753	Sp-E2f3	G1–S	+
		028827	Sp-E2f4	G1–S	+
		006312	Sp-DPI	G1–S	+
	Rb	011954	Sp-Rb	G1–S	+
		004292/025615	Sp-Rbl-1	G1–S	+
	P53	008978/008979	Sp-p53-like	G1–S	+
	Runx	006917/025612	Sp-Runt-1	G1–S	+
		007852	Sp-Runt-2	G1–S	−
Ubiquitin-proteolysis pathway	Cullin	002933	Sp-Cul-1	G1–S	+

(continued on next page)

Table 1 (*continued*)

Biochemical process/function	Family	Spu Id	Assigned name	Role of homologues	Embryonic expression
		026594	Sp-Cul-2	G1–S	+
		019174/021121	Sp-Cul-3	G1–S	+
		018555/001926/009731	Sp-Cul-4	DNA damage/replication	−
		006755	Sp-Cul-5		+
		018512	Sp-Apc2	Mitosis	+
	Fbox	019951	Sp-Fbw7	G1–S	−
		003152	Sp-Fbw7-like	G1–S	+
		019391	Sp-Skp2	DNA replication	+
		006781/007933	Sp-cyclin F	G2–M	+
		088809	Sp-Emi1	Mitosis	+
	Skp1	0024507	Sp-Skp1	G1–S	+
	Fzr/Cdh1	019392	Sp-Cdh1	Mitosis	+
	Fzy/Cdc20	004395	Sp-Cdc20	Mitosis	+
	Apc1	024153/008018/012580	Sp-Apc1	Mitosis	−
	Cdc27	022322	Sp-Apc3	Mitosis	+
	Apc4	021195	Sp-Apc4	Mitosis	+
	Apc5	011921	Sp-Apc5	Mitosis	+
	Cdc16	002193	Sp-Apc6	Mitosis	+
	Apc7	009240	Sp-Apc7	Mitosis	+
	Apc8	012696	Sp-Cdc23	Mitosis	+
	Apc10	025744	Sp-Apc10	Mitosis	+
	Apc11	021695	Sp-Apc11	Mitosis	+
Condensin/cohesin complexes	SMC1	021629	Sp-SMC1	Mitosis	+
	SMC2	024546	Sp-SMC2	Mitosis	+
	SMC3	019803	Sp-SMC3	Mitosis	+
	SMC4	013617	Sp-SMC4	Mitosis	+

[a] The embryonic expression (up to the mid–late gastrula stage) was deduced from the transcriptome analysis (Samanta et al., 2006). When the signal in the activity profile was negative or positive only for a minority of exons of a gene, the mRNA was considered as absent.

agreement with its proposed role in pre-mRNA splicing, the protein is localized to the nucleus (Genevière A.M., unpublished data). CDC2L5 appears to be the only gene of this type in *S. purpuratus*, suggesting that the two human kinases CDC2L5 and CrkRS represent a chordate or vertebrate-specific duplication.

While the functions of CDK/cyclin complexes regulating transitions between phases of the cell cycle or transcription are quite well understood, the binding partners and cellular role of some CDK-related proteins have still not been established. The PCTAIRE and PFTAIRE protein kinases are a subfamily of CDC2-related serine/threonine protein kinases named for a cysteine/phenylalanine-for-serine substitution in the PSTAIRE motif conserved in the initially characterized CDK proteins. Three PCTAIRE family genes have been identified in humans (Meyerson et al., 1992; Okuda et al., 1994), and two are found in mice (Okuda et al., 1992), whereas only one is present in *S. purpuratus*, as is the case in the cellular slime mold (Michaelis and Weeks, 1993). Similarly, a single PFTAIRE gene was identified in sea urchins.

CDK activating kinases and phosphatases

The CDK7/cyclin H complex, which phosphorylates the CTD of RNA-polymerase II, also participates in the full activation of CDKs by promoting phosphorylation of the conserved threonine residue within the T-loop region of these kinases (Kaldis, 1999). In metazoans, CDK7/cyclin H, in complex with the ring finger protein Mat1, is the major CDK activating kinase (CAK, reviewed in Harper and Elledge, 1998; Fisher, 2005). None of these proteins has been experimentally characterized in sea urchins; however, orthologues of cyclin H, CDK7 and Mat1 are found in the *S. purpuratus* genome, suggesting that the CAK complex is functional in sea urchins.

In yeasts, a second monomeric CAK activity has been reported (Espinoza et al., 1996; Kaldis et al., 1996; Lee et al., 1999; Thuret et al., 1996). Attempts to identify othologues of yeast CAK in the human genome were unsuccessful until the genome of *Drosophila* was used as intermediate in BLAST analysis, whereupon a protein (p42/Ccrk) with sequence similarity to both the CAK1 and CDK7 groups of CAK was discovered (Liu et al., 2004). However, its CAK activity in human remains controversial (Wohlbold et al., 2006). A homologue of p42/Ccrk is found in *S. purpuratus*. An analysis of *S. purpuratus* embryonic transcriptome (Samanta et al., 2006) indicates that this kinase is expressed during early embryogenesis, whereas neither Cdk7 nor cyclin H mRNA appears to be present. Given that Cdk7 inactivation causes embryonic lethality in *C. elegans* or *Drosophila* (Larochelle et al., 1998; Wallenfang and Seydoux, 2002), these results are intriguing. If the unexpected absence of CDK7/cyclin H mRNA in the embryo is verified, sea urchin embryogenesis may help to better understand the CAK activity requirement during embryonic cell cycles.

Cyclin B-associated CDK1 undergoes inhibitory phosphorylation at Thr 14 and Tyr 15 by the Wee1 family of protein kinases (Wee1, Mik1, and Myt1) and at entry into M phase, the inhibitory phosphates are removed by the activating phosphatase CDC25 (reviewed in O'Farrell, 2001). Structural orthologues of Wee1 and Myt1 have been identified in *S. purpuratus*, with high

similarity with the corresponding starfish *Asterina pectinifera* genes (Table S1, Nemer and Stuebing, 1996; Okano-Uchida et al., 1998; Okumura et al., 2002). Vertebrates have three CDC25 homologues (CDC25A, B and C), whereas only one was found in *S. purpuratus*, similar to its homologue in *A. pectinifera* (Deshimaru et al., 2002). Of the three vertebrate CDC25 paralogues, sea urchin CDC25 is most similar to CDC25B.

Mitotic kinases

The process of mitosis is controlled by phosphorylation events performed not only by CDK1 but also by other serine/threonine kinases, known as mitotic kinases. These kinases include three intensely studied proteins: the polo-like kinases (PLKs), the Aurora-related kinases and the NIMA-related kinases (NEK) (Nigg, 2001).

The founding member of the PLK family, Polo, was originally identified in *D. melanogaster* where it was shown to be required for mitosis (Fenton and Glover, 1993) and to have an activity peaking cyclically at anaphase–telophase (Glover et al., 1996). Plks have since proven to be important regulators for centrosome duplication and maturation, DNA damage checkpoint activation, mitotic onset, bipolar spindle formation, Golgi fragmentation, chromosome segregation, and cytokinesis (Barr et al., 2004; Blagden and Glover, 2003; Dai and Cogswell, 2003). Polo remains the single gene product of this family described in *Drosophila*, while four proteins sharing significant homology with Polo were identified in mammalian cells (Dai, 2005). The *S. purpuratus* genome encodes three Polo homologues (Table 1) as does the *C. elegans* genome (Chase et al., 2000; Ouyang et al., 1999), the *S. purpuratus* Plk1 being highly similar to the starfish orthologue (Table S1, Okano-Uchida et al., 2003).

The Aurora kinases also play a crucial role in regulating G2–M phase progression (Andrews et al., 2003; Eyers and Maller, 2003; Marumoto et al., 2005). The *S. purpuratus* genome apparently contains a single kinase in this family. The *C. intestinalis* genome similarly includes a single gene (Brown et al., 2004), whereas two genes have been described in *D. melanogaster* and *C. elegans*. Mammals alone have three Aurora kinases, Aurora A, B, and C, the two latter having

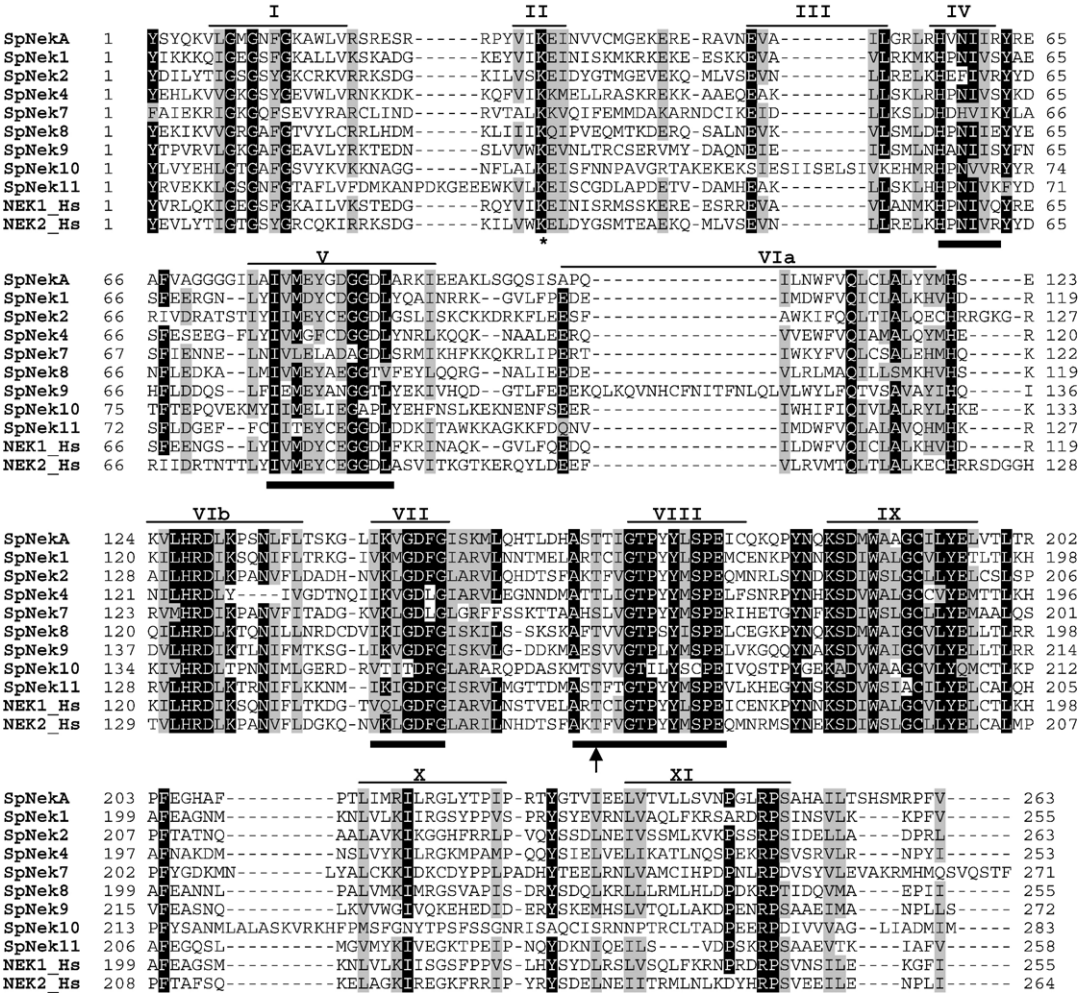

Fig. 3. Amino acid comparison of the kinase domain of the nine *S. purpuratus* NIMA-related kinases, with human Nek2 and Nek1. The eleven kinase subdomains are indicated in roman numerals, and diagnostic motifs for Nek family (Lu and Hunter, 1995) are underlined with bold bars. The star indicates the key lysine residue for kinase activity. Arrow points to a conserved serine/threonine residue, whose phosphorylation is required for activation. Identical residues are in black, conserved residues are in gray.

recently evolved from an ancestral Aurora B/C found in cold-blooded vertebrates (Brown et al., 2004). Aurora A is emerging as a critical regulator of centrosome and spindle function, while Aurora B mediates chromosome segregation by ensuring proper biorientation of sister chromatids. Aurora C, a chromosomal passenger protein similar to Aurora B, is specifically expressed in testis, indicating a role in spermatogenesis. The sequence of the sea urchin Aurora is very similar to that of its homologue in the starfish *Marthasterias glacialis* (Lapasset et al., 2005); however, like the two Auroras previously found in *C. elegans* and *D. melanogaster*, both sequences are highly divergent from the chordate counterparts (Brown et al., 2004).

The NEK proteins have been called the "third family of mitotic kinases" (O'Connell et al., 2003). The NEKs are represented in a wide variety of eukaryotic organisms, ranging from one to twenty or more representatives per genome. The NEK family possesses a highly conserved kinase domain at the N-terminus and a divergent C-terminus responsible for their specific roles in the cell. The founding member of the family, the NIMA kinase, is required for mitotic entry and progression through mitosis in *Aspergillus nidulans*. In higher eukaryotes, NEK2, the closest relative to NIMA, plays important roles in centrosome regulation, and its overexpression leads to centriole splitting and its association with centrosome-bound proteins (Fry, 2002). The functions of other members of the NEK family are less well understood.

Nine members of the NEK family are encoded in the sea urchin genome, the kinase domains of which are aligned in Fig. 3. While eight of them clustered with the identified mammalian NEK subfamilies (Table 1, Fig. 4), no orthologue could be found for NEK3 and NEK5. The ninth sea urchin NIMA-related kinase, Sp-NEKA, displays similarity with NEK1 in its kinase domain (Fig. 4). Sp-NEKA is highly related to a sea urchin *S. granularis* cDNA (AJ841701), however, no orthologue was found in other organisms, suggesting that Sp-NEKA may constitute an echinoderm-specific innovation of the NEK family.

Quarmby and Mahjoub (2005) showed a correlation between the number of NEK kinases in the genome of an organism and

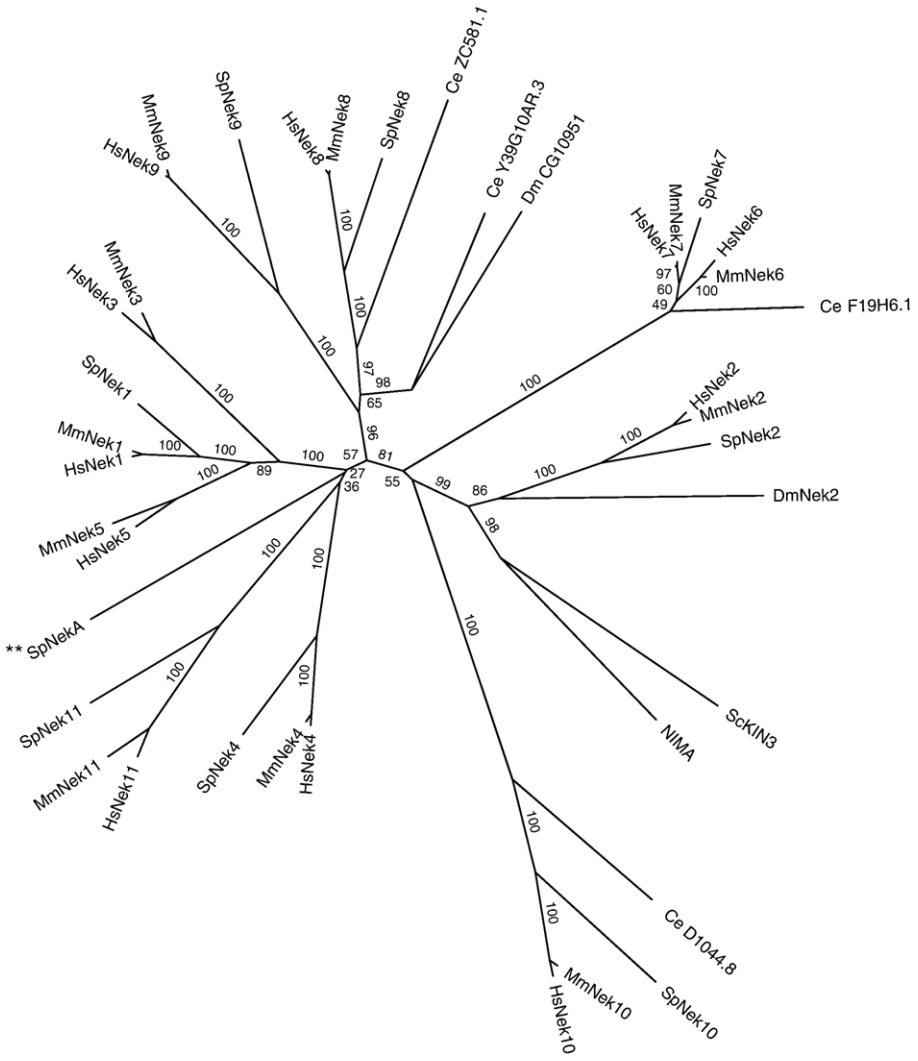

Fig. 4. Phylogenetic tree of NEK kinases. The kinase domains of NIMA-related kinases from *S. purpuratus* (Sp), human (Hs), mouse (Mm), *Drosophila melanogaster* (Dm), *Caenorhabditis elegans* (Ce), and *Aspergillus nidulans* (NIMA) were identified by ScanProsite program or retrieved from the kinome dataset at Kinase.com. (**) the sea urchin specific NEKA.

whether or not it has dividing ciliated cells. They proposed that the expansion of the NEK family has coevolved with centrioles, which serve both as basal bodies and microtubule-organizing centers. The complexity of NEK kinases found in *S. purpuratus* fits this hypothesis.

Checkpoint kinases

A complex network of checkpoint pathways helps ensure precise replication and segregation of the genome by delaying cell cycle progression in response to DNA damage or replication defects, facilitating repair of the defects. Genotoxic stress arising from unreplicated DNA or from DNA damage activates DNA damage checkpoint kinases such as ATM (ataxia telangiectasia mutated) and ATR (ataxia telangiectasia and Rad3 related) (reviewed in Sancar et al., 2004). These proteins in turn activate Ser/Thr checkpoint kinases, Chk1 and Chk2, and the p53 tumor suppressor, leading to inactivation of cyclin-dependent kinases to inhibit cell progression from G1 to S (the G1–S checkpoint) or from G2 to mitosis (G2–M checkpoint) (Sancar et al., 2004; Zakian, 1995). Orthologues of the key proteins involved in both the G1–S and G2–M DNA damage checkpoints were found in *S. purpuratus* (Table 1), including one isoform each of ATM, ATR, Chk1, and Chk2. Chk1 mRNA has also been identified in *S. purpuratus* eggs by RT-PCR (Adams, unpublished data).

The CDC25 phosphatases, which trigger the final activation of the CDK/cyclin complexes, are important downstream effectors of the Chk1/Chk2 response. Depending on the cell cycle stage and the nature of the damaging agent, activated Chks phosphorylate one or the other human CDC25 isoform, promoting either ubiquitin/proteasome mediated degradation (CDC25A in G1–S or S phase checkpoint) or 14-3-3 mediated sequestration (CDC25A and C in G2–M checkpoint) of the targeted phosphatase, preventing the activation of the downstream CDK/cyclin complex (reviewed in Kristjansdottir and Rudolph, 2004; Sancar et al., 2004; Stanford and Ruderman, 2005). As mentioned above, sea urchins have a single CDC25 homologue. Three 14-3-3 genes were identified in the *S. purpuratus* genome, whereas mammals have seven (Boston and Jackson, 1980). The 14-3-3 proteins play a pivotal role in several checkpoints targeting not only the CDC25 phosphatase but also CDK2, CDK4, and the CDK inhibitor p27 at the G1–S transition, and CDK1, Wee1, Chk1, and p53 at G2–M (Hermeking and Benzinger, 2006). How specificity of interaction among 14-3-3 isoforms is generated is not entirely clear, and 14-3-3 function in the unperturbed cell cycle is still poorly understood. As the three 14-3-3 genes are expressed during early embryogenesis (as indicated by the transcriptome analysis; Samanta et al., 2006), the sea urchin embryo should provide a good model for investigating their function.

The control of DNA replication

During S phase, the many thousands of replication forks involved in replicating chromosomal DNA must be coordinated to ensure that, despite the very large quantities of DNA involved, chromosomal DNA is replicated once and only once per cell cycle. Cells achieve this by having distinct stages that proceed sequentially. Prior to S phase, replication origins are "licensed" by loading pre-RC onto them. The licensing of replication origins depends on the sequential recruitment to DNA of ORC, Cdc6, Cdt1 and MCM2–7 proteins (Blow and Dutta, 2005; Forsburg, 2004; Nishitani and Lygerou, 2002) The six subunits of the ORC initiator (ORC1–6) were identified in the *S. purpuratus* genome, as were the six MCM helicases. Cdt1 and Cdc6 are also present (Table 2). Recently, two additional members of the MCM helicase family were identified: MCM8 and MCM9 (Gozuacik et al., 2003; Lutzmann et al., 2005; Yoshida, 2005). While MCM8 has been proposed to function as DNA helicase with a role distinct from that of the MCM2–7 complex, MCM9 function is unknown. MCM9 is more closely related to MCM8 than to the other MCM2–7 proteins and was hitherto known only in vertebrates, and thought to be a recent duplication of the MCM8 gene (Maiorano et al., 2006). The identification of MCM9 orthologues in the genome of *S. purpuratus* and the tunicate *C. intestinalis* (Fig. 5) demonstrates that the gene is not limited to vertebrates, suggesting that the MCM8/9 duplication occurred within deuterostomes prior to the split between chordates and echinoderms.

Table 2
S. purpuratus DNA replication genes identified in this study

Biochemical process/function	Family	Spu Id	Assigned name	Embryonic expression
Components of pre-replication complexes (pre-RC)	ORC	019821	Sp-ORC1	+
		010533	Sp-ORC2	+
		000544	Sp-ORC3	+
		024094	Sp-ORC4	+
		018823	Sp-ORC5	+
		005343	Sp-ORC6	+
	MCM	06096/011491	Sp-MCM2	+
		012983/006848	Sp-MCM3	+
		024515	Sp-MCM4	+
		012431	Sp-MCM5	+
		023819	Sp-MCM6	+
		026450	Sp-MCM7	+
	CDC6	010595	Sp-CDC6	+
	Cdt1	002046	Sp-Cdt1	+
Inhibitor of pre-RC	Geminin	023715	Sp-Geminin	+
Components of replisome progression complexes	CDC45	023032/024816	Sp-CDC45	+
	RPA	028925	Sp-RPA1	+
		026990	Sp-RPA2	+
		019078	Sp-RPA3	+
	GINS	012376	Sp-Sld5	+
		017817/017818	Sp-Psf-1	+
		021616	Sp-Psf2	–
		018457	Sp-Psf3	+
	MCM-10	005667	Sp-MCM10	+
Polymerases (DNA-directed)	Alpha-1	000210	Sp-Pol-alpha-1	+
	Alpha2	011199	Sp-Pol-alpha-2	+
	Epsilon-1	025749	Sp-Pol-epsilon-1	+
	Epsilon-2	014691	Sp-Pol-epsilon-2	+
Regulators of initiation	CDC7	000046	Sp-CDC7	+
	Dbf4	023982	Sp-Dbf4	+
Unknown	MCM	007007	Sp-MCM8	+
		003633	Sp-MCM9	+

Activation by protein kinases is required for the initiation of licensed origins (Bell and Dutta, 2002). In higher eukaryotes, the activities of CDK2/cyclin E and Cdc7/Dbf4 have been shown to result in changes in the pre-RC that lead to the binding of Cdc45 to the MCM complex followed by the unwinding of replication origins and the recruitment of RPA, DNA polymerase α and ε to initiation sites. Replication initiation is tightly coupled to removal of the license of origins (reviewed in Blow and Dutta, 2005). Metazoans mainly prevent re-licensing during S phase and G2 by the inhibitory binding of geminin to Cdt1. Homologues of all the components of this DNA replication regulatory network were found in the *S. purpuratus* genome as reported in Table 2.

Additional levels of cell cycle regulation

Proliferative disorders such as cancer are associated with somatic mutations and genomic instability, which are generally caused by errors in DNA replication or mitosis. Premature entry into either S or M phase increases the probability of error, and hence multiple levels of cell cycle control machinery are dedicated to ensuring that this does not occur. In animals, these control networks are linked to the transcriptional regulatory networks that control development (reviewed in Coffman, 2004). In addition to being under the control of the cyclins, CDKs, and other regulatory machinery discussed above, G1–S phase and G2–M phase transitions are regulated by CDK inhibitors such as mammalian $p27^{kip1}$ and $p21^{cip1/waf1}$ and the INK4 proteins and by expression levels and activities of transcription factors and cofactors such as E2F, pRB, and p53 family members (reviewed in Coffman, 2004; Sherr, 2000). Many of the CDKs discussed above function as switches in the proteolytic pathways that control levels of cyclins and other cell cycle control proteins, phosphorylated forms of which are targeted for proteolytic degradation by ubiquitin-ligases in the SCF and APC complexes (Reed, 2003). CDK inhibitors and regulators, transcription factors associated with cell cycle control, and proteins involved in ubiquitin-mediated proteolysis found encoded in the *S. purpuratus* genome are listed in Table 1. Notable absences include the INK4 CDK inhibitors, and the ARF tumor suppressor, which to date have been identified only in mammals and are probably vertebrate (and possibly mammalian) novelties. Moreover, the presence of two pRB family homologues, two E2Fs, a single p53 homologue, and one $p21^{cip1}/p27^{kip1}$ homologue in sea urchins mirrors the situation in flies, suggesting that this is the primitive bilaterian

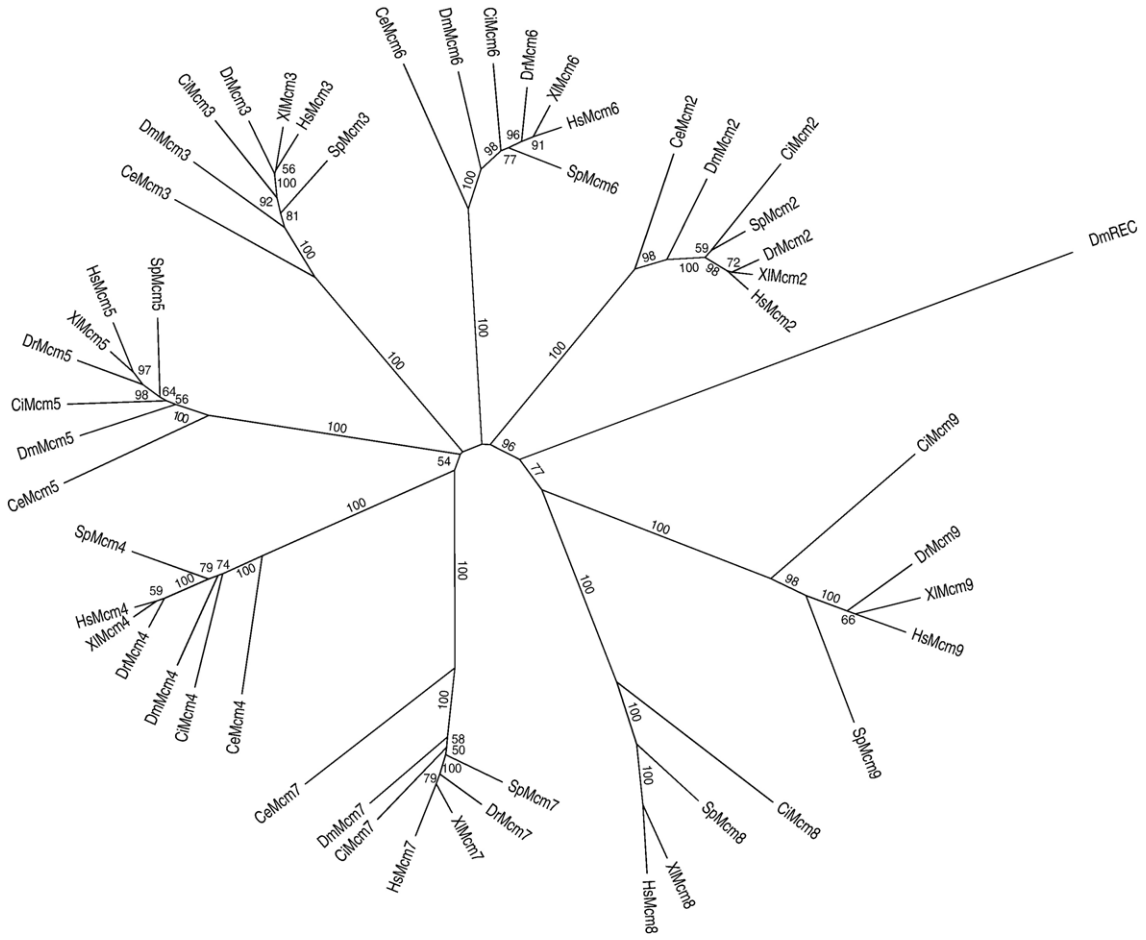

Fig. 5. Phylogenetic tree of MCM proteins. The tree was constructed by the Maximum Likelihood method (ML), with bootstrap values shown for each clade. Sequence Ids for the human (Hs), *Xenopus laevis* (Xl), *Danio rerio* (Dr), *Drosophila melanogaster* (Dm), *Caenorhabditis elegans* (Ce), and *Ciona intestinalis* (Ci) MCMs are reported in Table S3.

repertoire of these genes, which underwent expansion and diversification in the chordate–vertebrate lineage.

An extensive network of enzymes and other proteins that detect and repair damaged DNA provides another critical line of defense against mutation and genomic instability. Sea urchin genes associated with DNA repair functions (base excision, double-strand break, nucleotide excision, and mismatch repair) were identified using BLAST searches with mouse protein sequences retrieved by Gene Ontology searches (as described in Materials and methods); these are listed in Table S2. In general (and as might be expected), this set of genes is highly conserved between vertebrates and sea urchins. Homologues were found for all of the searched mammalian repair proteins, with the exception of one DNA base excision repair protein and two nucleotide excision repair proteins (Table S2).

Conclusions

The *S. purpuratus* genomic toolkit for cell cycle control is similar to, albeit somewhat simpler than, that of vertebrates. For most gene families, each subtype is represented by a single gene with some limited but notable exceptions. This holds particularly true for the CDK and cyclin families.

A comparison of sea urchin cell cycle genes with those from vertebrates, *C. elegans*, *Drosophila melanogaster*, as well as the tunicate *C. intestinalis*, provides new insight into the evolution of cell cycle control. While some gene subtypes have undergone lineage-specific expansions in vertebrates (i.e. the cyclins A, B, D, and E, the mitotic kinases aurora, polo, and NEK), others seem to be lost in that lineage (i.e. the novel cyclin B identified in *S. purpuratus* and *C. intestinalis* which are not found in zebrafish, mouse, or humans). On the other hand, the recently identified replicative helicase MCM9, which was previously thought to be a vertebrate innovation, is also found in sea urchins. Moreover, analysis of the sea urchin genome highlighted a few genes which either are specific to the echinoderm lineage, such as NekA, or were identified in *S. purpuratus* and found to have potential orthologues in other species, such as the novel family of cyclin K/L-related proteins. Most of the genes participating in the initiation and progression of DNA replication, in DNA repair, or in the tight control of cell cycle events, such as the checkpoint proteins, are highly conserved between echinoderms and vertebrates. The CDK inhibitors are a notable exception, with only one p21/p27 homologue, and an absence of the INK4 family, which is apparently confined to vertebrates.

Most of the genes included in this analysis (Tables 1 and 2) are expressed during embryogenesis (109/120) as deduced from the transcriptome analysis (Samanta et al., 2006), and those strictly related to cell cycle control are all being expressed at this stage. The genomic repertoire of cell cycle regulators uncovered in this survey will thus provide molecular tools that further enhance the physiological features that have made the early sea urchin embryo a productive model for understanding cell cycle control and its developmental regulation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2006.09.012.

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