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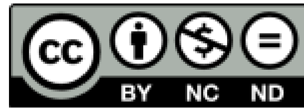

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Genes coding for cyclin-dependent kinase inhibitors are fragile in *Xenopus*

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ABSTRACT

Cell proliferation is strictly regulated by the dosage balance among cell-cycle regulators such as CDK/cyclin complexes and CDK-Inhibitors. Even in the allotetraploid genome of *Xenopus laevis*, the dosage balance must be maintained for animals to stay alive, and the duplicated homeologous genes seem to have gradually changed, through evolution, resulting in the best genes for them to thrive. In the *Xenopus laevis* genome, while homeologous gene pairs of CDKs are fundamentally maintained and a few cyclin genes are amplified, homeologous gene pairs of the important CDK-Inhibitors, *CDKn1c* and *CDKn2a*, are deleted from chromosomes L and S. Although losses of *CDKn1c* and *CDKn2a* can lead to diseases in humans, their loss in *X. laevis* does not affect the animals' health. Also, another gene coding *CDKn1b* is lost besides *CDKn1c* and *CDKn2a* in the genome of *Xenopus tropicalis*. These findings suggest a high resistance of *Xenopus* to diseases. We also found that *CDKn2c.S* expression is higher than that of *CDKn2c.L*, and a conserved noncoding sequence (CNS) of CDKn2c genomic loci on *X. laevis* chromosome S and *X. tropicalis* has an enhancement activity in regulating the different expression. These findings together indicate a surprising fragility of CDK inhibitor gene loci in the *Xenopus* genome in spite of their importance, and may suggest that factors other than CDK-inhibitors decelerate cell-cycling in *Xenopus*.
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1. Introduction

Cell proliferation is precisely controlled through regulation of the cell-cycle. Progression of the cell cycle is accelerated by the activation of cyclin-dependent kinases (CDKs) and decelerated or inhibited by inhibition of CDKs with associated CDK inhibitors (CDKIs) (Nigg, 1995; Sherr, and Roberts, 1995; Harper and Elledge, 1996; Nakayama and Nakayama, 1998). Since CDKIs act as regulators of CDKs, CDKIs are responsible for the regulation of cell proliferation in somatic cells. Some CDKIs are responsible for cell-cycle arrest at the G1 phase in somatic cells of higher eukaryotes via inhibition of CDKs, and thus are also involved in differentiation, for which G1 arrest is essential (Vidwans and Su, 2001). Therefore, CDKIs also have an important role in the regulation of development.

In general, the higher eukaryotes have seven CDKIs, which are divided into two families, CDKn1 (CIP/KIP) and CDKn2 (Ink4), based on their sequence homology and structures (Sherr and Roberts, 1995; Harper and Elledge, 1996). The CDKn1 family of proteins, which includes CDKn1a (p21cip1), CDKn1b (p27kip1) and CDKn1c (p57kip2), can bind to a wide variety of Cyclin-CDK complexes: their binding inhibits kinase activity of CDK4/CDK6 associated with D cyclins, CDK2 associated with either cyclin E or cyclin A, and CDK1 associated with cyclin A or cyclin B (Sherr and Roberts, 1995; Harper and Elledge, 1996; Pines, 1997). Thus, they have the potential to block cell-cycle progression at multiple points. Also, CDKn1 family proteins have roles in the assembly of cyclin and CDK though their specificity to bind to both cyclins and CDKs (Sherr and Roberts, 1999). In contrast to the CDKn1 family, CDKn2 family proteins, which include CDKn2a (p16ink4a), CDKn2b (p15ink4b), CDKn2c (p18ink4c) and CDKn2d (p19ink4d), exclusively inhibit cyclin D-dependent kinases CDK 4 and 6. Binding of a member of the CDKn2 family to CDK 4 and 6 keeps them from associating to cyclin D and activating (Sherr and Roberts, 1995, 1999; Roussel, 1999), leading to accumulation of hypophosphorylated pRb and G1 arrest of the cell cycle (Ewen, 1994; Lukas et al., 1995; Medema et al., 1995). Although CDKn2 family proteins directly inhibit CDK4 and 6, their upregulation induces indirect inhibition of CDK2 activity through a redistribution of CDKn1 associated withCDK4/6 to CDK2 (Poon et al., 1995;

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Reynisdóttir et al., 1995; Han et al., 2005).

In this study, we analyzed the genes coding for cell-cycle regulators, especially CDKIs, in the allotetraploid genome of *Xenopus laevis*. The analysis showed that genes coding for CDKn1c and CDKn2a are lost in both homeologous loci, and a gene classified into the CDKn1 family novelly appears. Also, another gene coding *CDKn1b* is lost only in the genome of *Xenopus tropicalis*, indicating that this gene-loss occurred after the speciation of *X. tropicalis*, 48Mya (million years ago). We also found that alterations in the coding and non-coding sequences of *CDKn2c* lead to differences of their function and expression between CDKn2c.L and CDKn2c.S. These findings together indicate a high fragility of the CDKI genes in the genome of *Xenopus*.

2. Material and methods

2.1. Analysis of gene structures in X. laevis and their gene expression

Analysis of the gene structures coding for cell-cycle regulators was performed based on the gene model of *X. laevis* J-strain ver 9.1. Their expression was analyzed with transcriptome data for seven developmental stages and fourteen tissues, which were obtained from RNA-seq experiments (Session et al., 2016). In brief, RNA-seq experiments were performed as follows: RNAs isolated from embryos and tissues were used for construction of cDNA libraries with the standard non-strand specific mRNA library preparation protocol using Truseq RNA sample prep kit (Illumina). These were subjected to paired-end sequencing, and the obtained datasets of more than 1 billion RNA-seq reads were used for the analysis.

2.2. Preparation of cell lysates

HeLa cells were seeded at a density of 2.5×10^5 in 6-well plates and cultured for 1 d. Transfection of each plasmid was performed with transfection reagent (Fugene 6; Promega). After 1 d, the cells were extracted for lysate production as described previously (Han et al., 2005).

2.3. Exploring cis-regulatory candidates for cdkn2c expression

To explore *cis*-regulatory candidates for enhancers of *cdkn2c*, we initially compared genomic sequences of a 14-kb segment encompassing *cdkn2c* in *X. tropicalis* with its orthologous regions in *X. laevis L* and *S* using the MultiPipMaker alignment tool (Schwartz et al., 2000). The following sequences from the National Institute of Genetics (NIG) were used for alignment: xenTro2-scaffold_1_4931215-4945545, cdkn2c_L_DNA+Clones+JGI_gene_v1_4_Scaffold53263_1780616..1797615, cdkn2c_S_DNA+Clones+JGI_gene_v1_4_Scaffold69443_4764000..4778999.

The open-access database JASPAR ver. 5 was used to search for potential transcription factor-binding sites (Mathelier et al., 2014). CNSs were aligned using ClustalW, and conserved transcription factor-binding sequences were further analyzed by phylogenetic footprinting (Blanchette and Tompa, 2002). A genomic element (CNE) conserved in the *cdkn2c* loci between *X. tropicalis* and *X. laevis S* was amplified from *X. laevis* J-strain and *X. tropicalis Nigerian* genomic DNA by PCR and cloned into *Sac*II and *Xba*I sites of a reporter plasmid, β-GFP (Ogino and Ochi, 2009), which carries GFP regulated by a chicken β-actin basal promoter. The primer sequences used in this study were:

Xt_cdnk2c-CNE-F: tccgcggGAGAACTAGAATGGGCGCCCAAATCG
Xt_cdnk2c-CNE: gctctagaGCCTGTCTATAAAGCTGCATAATGTGGC
Xl_cdnk2c-CNE-S: atccgcggCACATTCGAACTGCACGCCCTGATAC
Xl_cdnk2c-CNE-S: gctctagaGATTTGACTAGCGCAAAACTGCGCAAAAAC.

Primer linker sequences are shown in lowercase letters. The locations of the *cis*-regulatory elements in the genome assemblies are in Chr04:85960521..85960766 (*X.tropicalis* ver. 9) and chr4S:69927237..69927451 (*X. laevis* J-strain ver. 9.1), respectively. Each reporter plasmid was used for generation of transgenic frog embryos according to the sperm nuclear transplantation method, as previously described (Tanaka et al., 2003; Suzuki et al., 2015). The manipulated embryos were cultured and normal embryos were subjected to in situ hybridization to detect precise expression of GFP. We performed the reporter assays together with a control reporter, which has no enhancer region, and confirmed the specificity of the reporter-gene expression regulated by the enhancer.

2.4. Antibodies, immunoprecipitation and immunoblotting

Antibodies against cdk4 (H-22) and flag (M2) were obtained from Santa Cruz and Sigma-Aldrich, respectively. Immunoprecipitation was performed as described in Shirako et al. (2008). Immunoblotting was performed as described previously (Ushio et al., 2009).

2.5. Comparison of developmental speed between X. laevis and X. tropicalis

Thirty healthy eggs of *X. laevis* (J-strain, 1.4-mm diameter) and *X. tropicalis* (Nigerian strain, 0.9-mm diameter) were selected, respectively, and simultaneously subjected to artificial fertilization with sperm of each species in individual cell-culture dishes. Both fertilized eggs were cultured in a single dish at 26 °C. Times (hours) after fertilization were determined at the indicated stages until stage 42.

3. Results

3.1. Genes coding for CDKs, cyclins and other cell-cycle regulators are fundamentally retained even after allotetraploidization

Based on a genomic analysis of *Xenopus laevis*, the L and S subgenomes are estimated to have diverged from each other 34 Mya in the *X. laevis* ancestors, and the allotetraploidization is estimated to have occurred 17–18Mya (Session et al., 2016). Since genes coding for cell-cycle regulators are essential for cell proliferation, each ancestral genome of *X. laevis* had a set of genes for cell-cycle regulation. The allotetraploidization that occurred in *X. laevis* resulted in full duplication of the genes for cell-cycle regulation. Cell-cycles are fundamentally regulated by the dosage balance among the cell-cycle regulators including CDK/cyclin complexes and CDK-inhibitors (CDKIs) (Nurse, 2000). Since duplication of genes allows their potential maximum expression levels to be doubled, the homeologous gene pairs for cell-cycle regulation must be retained in order to maintain the dosage balance among cell-cycle regulators. Thus, we studied the structures of homeologous genes coding for CDKs, cyclins and other cell-cycle regulators on the *X. laevis* genome.

As expected, comparative analysis showed that most of the principal genes for cell-cycle regulators were retained as homeologous genes, and each retention rate in the two copies was higher than that of all genes (56%) (Fig. 1a and b) (Session et al., 2016). However, some exceptions were also found, especially in *cyclin* (*ccn*) genes (Table 1). In the *Xenopus* genome, both homeologous genes of cyclin D3 are lost, and a novel D-type cyclin (cyclin Dx) appears as a pair of homeologous genes. Although a homeologous gene of the novel cyclin D on chromosome L also has a deletion at its initial Met (data not shown), the appearance of the novel cyclin genes may compensate for the gene-loss of cyclin D3.

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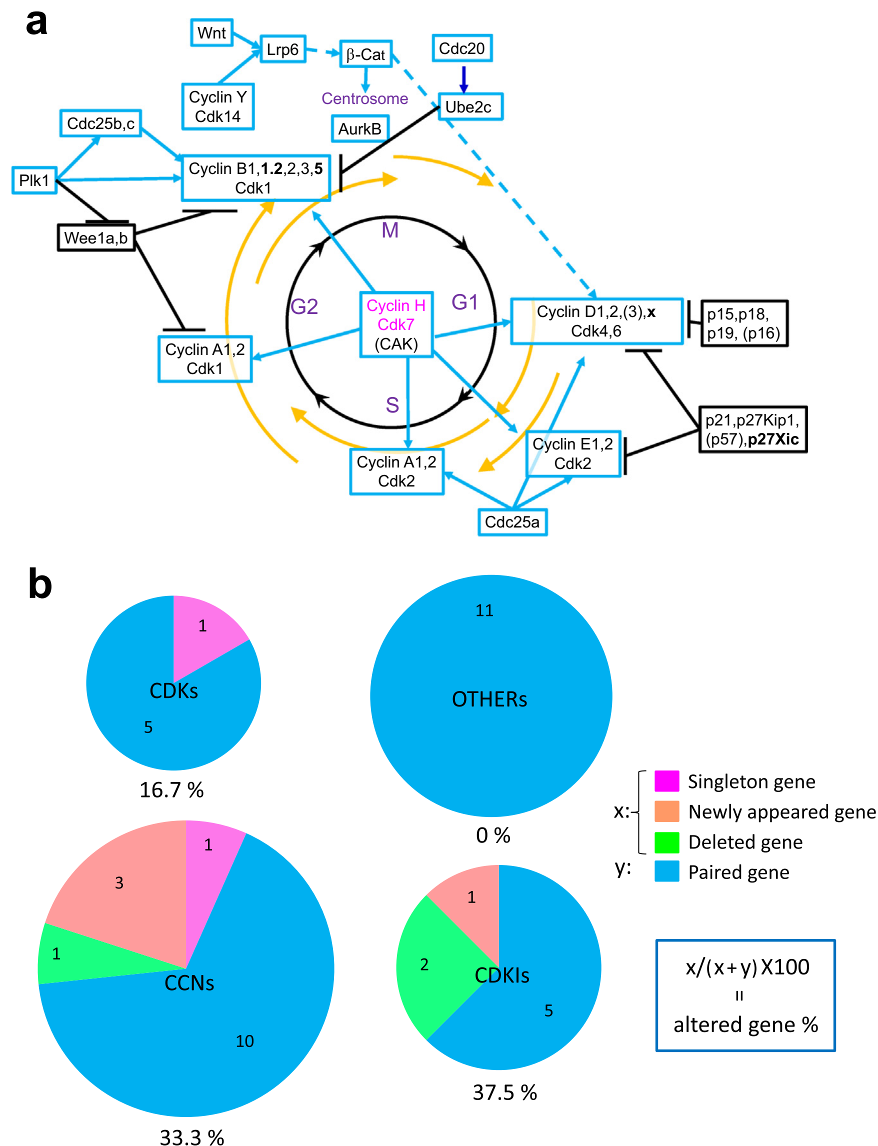

Fig. 1. Structures of genes involved in cell-cycle regulation in the *X. laevis* genome. (a) Summaries of gene structures. Most genes are retained as homeologous pairing genes. Black characters, a homeologous pairing gene; magenta characters, a singleton gene. Genes lost (ccd3, CDKn1c (p57), CDKn2a (p16)) and amplified (ccnb1.2, ccnb5, ccndx, CDKnx (p27Xic)) are given in parentheses and bold, respectively. Colored squares and lines represent a positive (blue) or negative (black) role in the cell-cycle. (b) Pie charts summarize the structures of genes categorized into each group. Numbers in each chart indicate genes subdivided into each category, and those out of charts indicate percentages of genes with altered structures.

Table 1
Summary of genes involved in cell-cycle regulation in *Xenopus laevis*. These Gene structures are also summarized in Fig. 1a.

	Singletons	Homeologous pairs	Newly-appeared genes	Deleted genes
CDKs	cdk7	cdk1, cdk2, dk4, cdk6, cdk14	0	0
CCNs	ccnH	ccnA1, ccnA2, ccnB1, ccnB2, ccnB3, ccnD1, ccnD2, ccnE1, ccnE2, ccnY	ccnDx ([a]), ccnB1.2 ([b]), ccnB5 ([c])	ccnD3
CDKIs	0	cdkn1a, cdkn1b, cdkn2b, cdkn2c, cdkn2d	cdknX ([d])	cdkn1c, cdkn2a
OTHERs	0	cdc25a, cdc25b, cdc25c, wee1a, wee1b, plk1, lrp6, b-cat, cdc20, ube2c, aurkB	0	0

[a] ccnDx.L has shorten N-terminus because of deletion of initial met.
[b] ccnB1.2 genes are homeologous.
[c] ccnB5 gene is singleton.
[d] N-termini of CdknX.L and CdknX.S are different.

As for B-type cyclin genes, a novel cyclin B gene, *cyclin B1.2*, appears as homeologous gene pairs on chromosome 4L and 4S, and another novel cyclin B, B5, also appears as a singleton on chromosome 3L (Table 1 and Fig. 1a). Since cyclin B1.2 has a high similarity to cyclin B1 (92%), it is plausible that cyclin B1.2 was amplified from cyclin B1, though they are on different chromosomes (cyclin B1.2 is on chromosome 4 and cyclin B1 is on chromosome 1). *Cyclin B5* gene seems to be tandemly duplicated from cyclin B2.L, since cyclin B5 gene is located next to cyclin B2.L on chromosome 3L. However, the similarity between them is low (62% in amino acids, 73% in nucleic acids). These findings indicate that genes coding for cyclins are relatively more changeable than those for their binding partner, CDKs, and other cell-cycle regulators (Fig. 1b). In contrast, *X.laevis* genome has a gene coding for CDK7 as a singleton (Table 1). It is known that CDK7 associates with cyclin H, leading to the CDK-activating kinase, CAK. Our analysis showed that its binding partner, cyclin H, is also a singleton gene (Fig. 1b and Table 1), suggesting that cyclin H is the only binding partner for CDK7, and that precise regulations of both

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Table 2
Comparisons of genes coding for CDKIs among species. Results were obtained by searching genes on each gene model and/or searching transcripts with the blast sequence analysis on databases in NCBI. ○, a gene which is retained. A number in parentheses in a *Xenopus* column shows a chromosome number confirmed by FISH. Dre, zebrafish; Tni, Tetraodon; Psi, Chinese softshell turtle; Cpi, painted turtle; Aca, green anole; Gga, chicken; Mmu, mouse; Cfa, dog; Bta, cow; Hsa, Human.

Table 2

			Xenopus										
			Xtr	*Xla*									
CKI genes	**Dre**	**Tni**		**L**	**S**	**Psi**	**Cpi**	**Aca**	**Gga**	**Mmu**	**Cfa**	**Bta**	**Hsa**
cdkn1a (p21CIP1)	○	○	○(2)	*1 ○	○(2S)	○	○	○	○	○	○	○	○
cdkn1b (p27KIP1)	○	○	nd	○	○(3S)	○	○	○	○	○	○	○	○
cdkn1c (p57KIP2)	○	○	nd	nd	nd	○	○	○	*5 ○	○	nd	○	○
cdknx (p27XIC1)	—	—	○(7)	*2 ○(7L)	*3 ○(7S)	—	—	—	—	—	—	—	—
cdkn2a (p16INK4a)	○	*8 △	nd	nd	nd	○	*5 ○	○	○	○	○	○	○
cdkn2b (p15INK4b)	○	*8 △	○(1)	○	○(1S)	*5 ○	*5 ○	○	○	○	○	○	○
cdkn2c (p18INK4c)	○	○	○(4)	*4 ○(4L)	○(4S)	*6 ○	○	○	○	○	○	○	○
cdkn2d (p19INK4d)	○	○	○(3)	○(3L)	○	*7 ?	○	○	*7 ?	○	○	○	○

○: a gene model or a transcript that includes ORF exists.

(shaded): amplified

nd: Not Detected (Deleted)

*1: mutations exist; *2: 56 aa from N′ are deleted (Fig. 2); *3: N′ is different from *X.tropicalis* (Fig. 2); *4: The expression and CDK4-binding ability is lower than those of CDKn2C.S (Fig. 3 and 5); *5: the sequence includes each CDKN-like protein; *6: The sequence includes a predicted protein; *7: the gene can not be identified because of many similar sequences in the database. *8: *cdkn2a* and *2b* are combined into one gene (*cdkn2a/b*).

genes are important for keeping *Xenopus* alive during development (see also the Discussion).

3.2. Two CDKI genes are deleted, and a novel CDKI gene appears in the Xenopus genome

While genes coding for cell-cycle regulators were fundamentally retained in two copies (Fig. 1 and Table 1), we found prominent exceptions in the family of genes coding for CDKIs (Fig. 1b and Table 1). In general, the CDKI gene family consists of seven genes in a wide variety of animals, including zebrafish, turtles, green anoles, chickens, mice, cows and humans (Table 2). Every CDKI gene has an essential role in the inhibition of cell proliferation, and deletion or loss-of-function of one of them leads to serious diseases including cancers (Sherr and Roberts, 1995, 1999; Roussel, 1999; Kato et al., 2001). Therefore, it is generally thought that genes coding for CDKIs must be conserved. In fact, the expression of four *CDKI*s included in CDKn1 and CDKn2 families has been reported in *Xenopus* (Su et al., 1995; Daniels et al., 2004; Doherty et al., 2014). However, we found exceptions in *Xenopus* and dogs (Table 2). Especially in the *X. laevis* genome, a comparative analysis surprisingly uncovered that the genes coding for CDKn1c (p57kip2) and CDKn2a (p16ink4a) are deleted from both chromosomes L and S. Instead, a novel CDKI gene, which is classified into the CDKn1 family, appeared in chromosomes 7L and 7S (Table 2). These alterations of CDKI genes are common in *X. tropicalis* (Table 2), and syntenies around the genes are conserved (data not shown) indicating that the alterations of CDKI genes occurred before the speciation of *X. laevis* and *X. tropicalis*, 48 Mya (Session et al., 2016). In addition, *X. tropicalis* also has an additional deletion of *CDKn1b* (p27kip1), indicating that this deletion occurred during 48 million years after the speciation of *X. tropicalis* . These findings together showed exceptional fragility of CDKI family genes in the *Xenopus* genome.

3.3. Retained genes coding for CDKIs are also fragile on the X. laevis genome

Since we found that some genes coding for CDKIs are deleted and an alternative gene appeared, it is possible that homeologous genes, which are retained in L or S chromosomes in *X. laevis*, are also altered. Comparative analysis of the gene coding for CDKnx,

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Xtr   1   MGIAPLVLDYNSQHPGPELGDVRKVKKGAAAPTPSPTLRIGVLPPSPLVSSSLLLFFPPL  60
XlS   1   ------------------------------------MATQQLVYINYSISSLLLLFFSPL  24
XlL       ----------------------------------------------------------

Xtr  61   SAPTPFLYKLPESLPRHIHIELSPAHTHSNHRAMAAFHIALQEEMI---PAALPRVSAGT  117
XlS  25   TTAAPFLYKLPKPLPRHIHIELS-THTQQSQQAMAAFHIALQEEMIVASPAALPRLSLGT  83
XlL   1   ---------------------------------MAAFHIALQEEMISA-PAVLPRLSAGT  26

Xtr 118   GRGACRNLFGPIDHDELRSELKRQLKEIQASDCQRWNFDFESGTPLKGIFCWESVESKDV  178
XlS  84   GRGACRNLFGPIDHDELRSELKRQLKEIQASDCQRWNFDFESGTPLKGTFCWEPVETKDV  143
XlL  27   GRGACRNLFGPIDHDEMRSELKRQLKEIQASDCQRWNFDFETGTPLKGIFCWEPVESKDM  86

Xtr  179  PTFYSQNRSSAANTTTPSRQQQPLLVSRQPEPREEAPLDTVRNVPNPPCAKENAEKTIKR  238
XlS  144  PSFYSPSRSLATNTTPQSRQQQPLLVSRQPEPREEAPVDTVRNVPNPPCAKENAEKIIKR  203
XlL  87   PSFYSQNRSIAANTTPSPRQQQPLLVSRQPEPREEAPVDTVRNVPNPPCAKENAEKTVKR  146

Xtr  239  CQGVKGPAKASAIPSTQHRKREITTPITDYFPKRKKILGAKPDATKGAHLLCPLEQTPRK  206
XlS  204  CQGVKGPTKASANTSTQRRKREITTPITDYFPKRKKILSAKPDATKGVHLLCPLEQTPRK  236
XlL  147  CQGVRGPAKASANTSTQRRKREITTPITDYFPKRKKILSAKPDATKGAHLLCPLEQTPRK  179

Xtr  207  KIR  300
XlS  237  KIR  266
XlL  180  KIR  209
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Fig. 2. Comparisons of amino acid sequences of CDKnx (Xic1) among *X. tropicalis* (Xtr) and chromosomes L (XlL) and S (XlS) of *X. laevis*. Alignments were calculated from data based on the gene models (*X.tropicalis* ver. 9, *X. laevis* J-strain ver. 9.1) using BLAST *Xenopus* (Xenbase). CDKnx on *X. laevis* 7L was originally reported as Xic1 by Su et al. Each N terminus, which does not affect the activity as CDKI, is not conserved among them.

which newly appeared on the *Xenopus* genome, showed that the lengths of coding sequences are different between homeologues on chromosomes 7L and 7S, because of differences in the initial Met (Fig. 2); the position of the initial Met for CDKnx.S is more upstream than that for CDKn.L. It is worth noting that the position of the initial Met for the *CDKnx* gene on *X. tropicalis* is also different from both *CDKnx* genes on *X. laevis* (Fig. 2). Besides, these three CDKnx genes have some different amino acid sequences in their common coding regions. The appearance of the CDKnx gene is specific for *Xenopus* (Table 2), indicating that the novel CDKn gene appeared after the speciation of *Xenopus*. Also, since the ancestral L and S subgenomes diverged from *X. tropicalis* 48 Mya and from each other 34 Mya (Session et al., 2016), each alteration in the coding sequences of CDKnx genes, which is specific to *X. tropicalis*, and chromosomes L and S of *X. laevis*, occurred independently during relatively short periods during evolution. This shows that many homeologous genes coding for CDKIs that are retained in L or S chromosomes in *X. laevis* are also fragile.

We also analyzed the expression of each CDKI gene, and found that CDKn2b and CDKn2c are differentially expressed between chromosome L and S: transcriptome data especially showed that CDKn2c.S is more strongly expressed than CDKn2c.L in embryos at stages 8-10 and in some organs such as the eye and testis (Fig. 3). As it is possible that the differing expression reflects alteration of regulatory regions on the genome, we next studied the non-coding regions responsible for the different expressions. Since *X. tropicalis*, the genome sequencing of which has been finished, also has a CDKn2c gene, we compared the genomic loci of CDKn2c.L and CDKn2c.S of *X. laevis* with that of *X. tropicalis*, and found conserved non-coding sequences (CNSs). While almost all CNSs were common among three genomic loci of *CDKn2c* in *X. laevis* and *X. tropicalis*, one CNS was lost only in the CDKn2c locus on chromosome 4L in *X. laevis* (Fig. 4a). Transgenic reporter analysis using this CNS showed that the reporter gene was expressed at eyes at which CDKn2c.S is up-regulated (Figs. 3b and 4b), indicating that this CNS has an enhancer activity responsible for the organ-specific expression of CDKn2c. These results showed that the fragility of CDKn genes in *X. laevis* exists in both coding regions and non-coding regions. In addition, we found that CDKn2c.L has substitutions of conserved amino acids, which seem to be associated with CDK4/6 (Fig. 5a; Russo et al., 1998). Immunoprecipitation

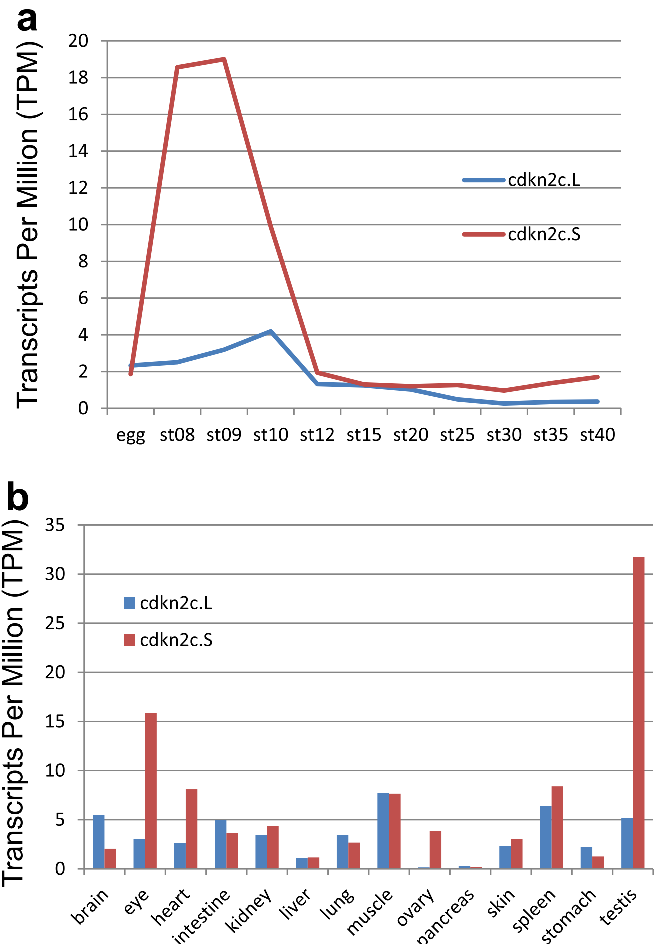

Fig. 3. Expression profiles of *cdkn2c* genes from L and S chromosomes. Abscissa indicates developmental stages (a) or adult tissues (b). RNA-seq data generated in duplicate for seventeen developmental stages and fourteen adult tissues were used for calculations of TPM, and construction of each graph (Session et al., 2016). The ordinate indicates TPM values. RNA-seq experiments were done twice with similar results, and representative data are shown.

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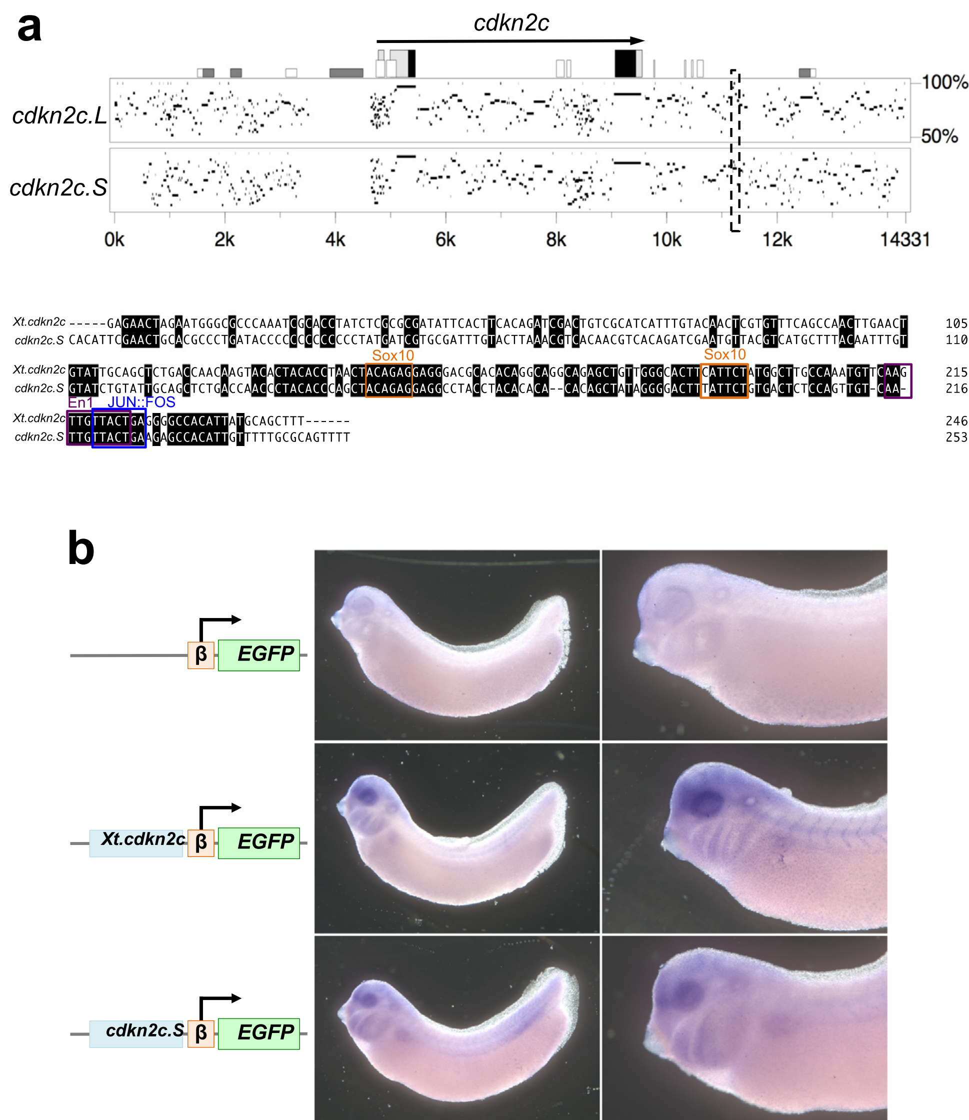

Fig. 4. Identification of *cis*-regulatory sequences, which contribute to the *cdkn2c.S* unique expression. (a) A comparison of non-coding sequences in *cdkn2c* genes among *X. laevis* chromosomes L and S and *X. tropicalis*. Upper panel: a genomic locus surrounded by dotted line indicates the conserved non-coding sequences (CNSs), which are retained in *cdkn2c.S* of *X. laevis* and *cdkn2c* of *X. tropicalis*, but are different from *cdkn2c.L* of *X. laevis*. Lower panel: sequences conserved between *cdkn2c.S* in *X. laevis* and *cdkn2c* in *X. tropicalis* are surrounded by black squares, and predicted binding motifs for transcription factors are indicated. (b) The CNSs in (a) were used for transgenic reporter analysis with *X. laevis*.

analysis with flag-CDKn2c.L and flag-CDKn2c.S, which were introduced into HeLa cells, showed that the binding ability of CDKn2c.L to CDK4 was much weaker than that of CDKn2c.S (Fig. 5b). These data suggest that CDKn2c.L tends to lose its function as a CDKI.

4. Discussion

With comparative analysis of *X. laevis* genes coding for cell-cycle regulators, we found that some genes for cyclins are amplified (Fig. 1a and b, Table 1). Cyclins are required for the regulation of cell proliferation through activation of CDKs (Nurse, 2000; Brooks and Thangue, 1999). Thus, amplification of cyclin genes allows their potential maximum expression levels to be high, leading to acceleration of cell proliferation. However, activation of CDKs requires not only association with cyclins, but also phosphorylation at a conserved residue in the T-loop by CAK (Lolli and Johnson, 2005). Inactivation of CAK or mutation of the phosphoacceptor residue of the target CDK leads to suppression of CDK activation, resulting in suppression of cell-cycle progression and arrest of development (Larochelle et al., 1998; Wallenfang and Seydoux, 2002). CAK is a heterodimer of CDK7 and cyclin H. In the *Xenopus* genome, we found that both CDK7 and cyclin H genes become singletons (Fig. 6), potentially leading to limitation of the amount and activity of CAK. While some cyclin genes are amplified in the *X. laevis* genome, it is possible that the potential limitation of CAK suppresses proliferation of cells accelerated by amplified cyclins (Fig. 1a). The ratio of the potential expression level of CDKs/cyclins to CAK is higher in *X. tropicalis* than in *X. laevis*, since the genes coding for CDKs and cyclins are retained on the genome of *X. tropicalis* and *X. laevis* except CDK7 and cyclin H on chromosome S of *X. laevis*. In fact, the expressions of *CDK7* and *cyclin H* are much lower in *X. laevis* than in *X. tropicalis*, while expression of other *CDKs* and *cyclins* are not fundamentally different or rather higher in *X. laevis* than in *X.tropicalis* (Yanai et al., 2011). These results may explain a feature of organisms with tetraploid genomes: they have a larger body size than those with diploid genomes (e.g., *Xenopus*, trout, plants; Fig. 7a; Lou and Purdom, 1984; Griffiths et al., 2000). In fact, the body length of *X. laevis* is 2.55 times that of *X. tropicalis*

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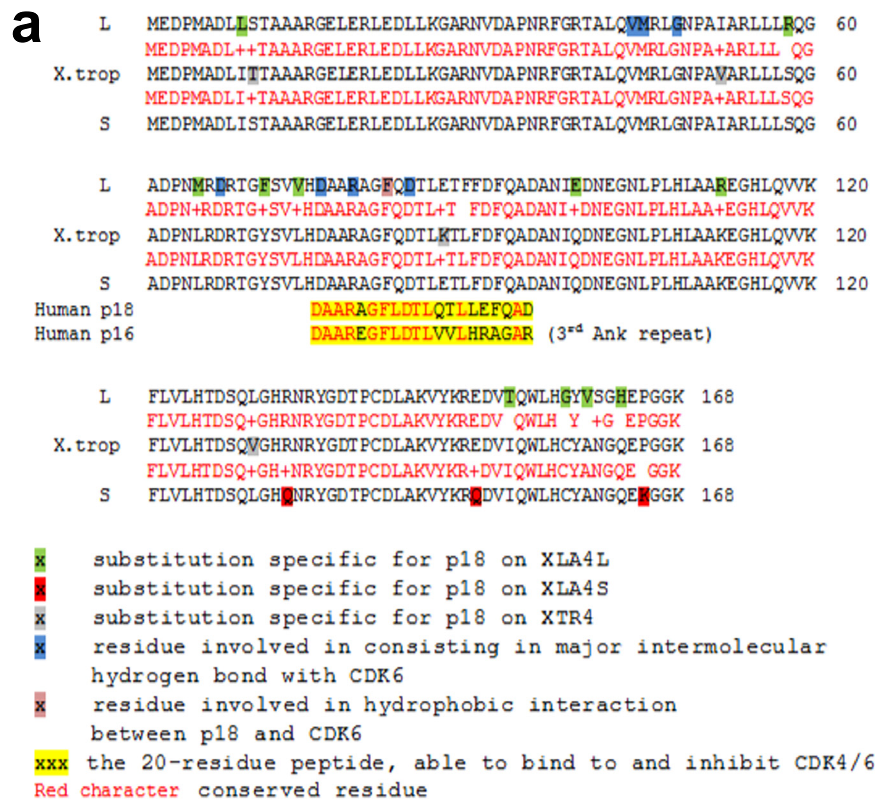

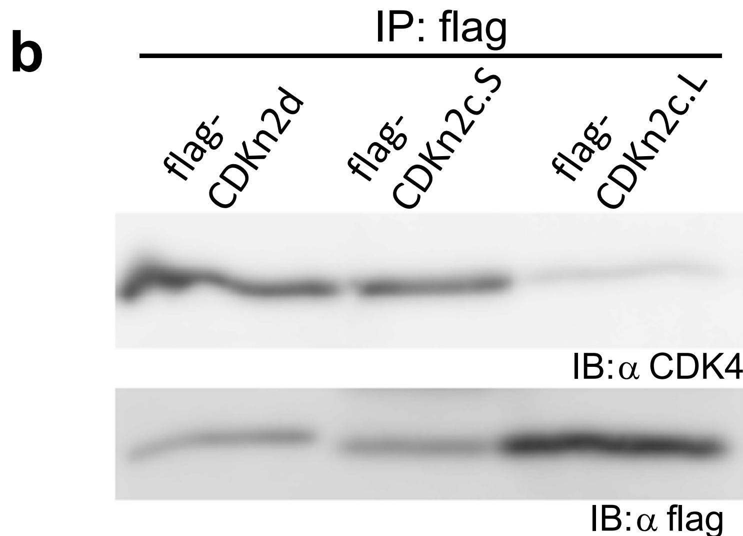

Fig. 5. Comparisons of binding abilities of CDKn2c.L and CDKn2c.S to CDK4. (a) Amino acid alignments among CDKn2c of *X. tropicalis* and CDKn2c.L and CDKn2c.S of *X. laevis*. The twenty residues responsible for association and inhibition for CDK4 in CDKn2a (p16) and CDKn2c (p18) of human are also shown. Alterations of amino acids are colored as indicated in (a). (b) Co-immunoprecipitation assay to compare binding abilities to CDK4 between CDKn2c.L and CDKn2c.S. Lysates were prepared from cells expressing the indicated protein, and used for immunoprecipitation with anti-flag antibody. The samples were subjected to immunoblotting with anti-CDK4 (upper) and anti-flag antibodies (lower), respectively. Experiments were done twice with similar results, and representative data are shown.

(Kobel and Tinsley, 1996), and this is caused in part by larger cell sizes in *X. laevis* than in *X. tropicalis* (Edens and Levy, 2014). It was reported that larger cell sizes in eukaryotes can be induced by slower progression of the cell-cycle (Morin et al., 2006). Since phosphorylation of CDKs by CAK is essential for cell-cycle progression (Larochelle et al., 1998; Lolli and Johnson, 2005), the lower expression of CAK possibly leads to slower progression of the cell-cycle in *X. laevis* than in *X. tropicalis*. Therefore, becoming singletons on *CDK7* and *cyclin H* genes may cause the larger body size in *X. laevis* than in *X. tropicalis*. In addition, the results showing lower expression of CAK in *X.laevis* than in *X. tropicalis* may explain the difference in developmental speed between *X. laevis* and *X. tropicalis*: the development of *X. laevis* is slower than that of *X. tropicalis* at each optimal temperature (16–22 °C for *X. laevis* and 25–30 °C for *X. tropicalis*; Xenbase, 2005). We also showed that the developmental speed of *X. laevis* after the maternal-zygotic transition (MZT) is slower than that of *X. tropicalis* even at the same temperature, 26 °C (Fig. 7b and c). However, more detailed analysis is required because the information available about their developments is not sufficient: we also need the cell numbers to

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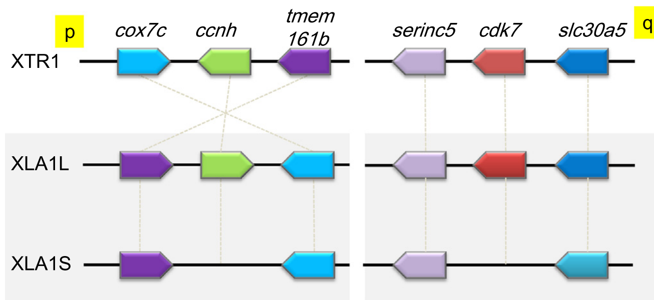

Fig. 6. Syntenies of *cdk7* and *ccnh* loci in *X. tropicalis* and *X. laevis*. Abbreviations for species and chromosome numbers: *X. tropicalis* (XTR1), *X. laevis* (XLA1L and XLA1S). The p- and q-arms of chromosome 1 are denoted by letters in the figure, respectively.

construct each embryo at each stage, and direct comparison of their cell-cycle speed (not developmental speed) especially after the MZT. Also, we can not rule out the possibility that higher temperature than the optimal temperature could damage some enzymes of *X. laevis*, leading to the slower progression of development in *X. laevis* than in *X. tropicalis* (Fig. 7b and c). In fact, the temperature, 26 °C, allows the heat shock promoter to be activated in embryos of *X. laevis* (Harland and Misher, 1988). Despite these points that require attention, the lower expression of *CDK7* in *X. laevis* than in *X. tropicalis* may have more effects on cells and animals than expected, since CDK7 has another role in the general transcription factor TFIIH as a kinase subunit, and is required for transcription through phosphorylation of the C-terminal domain (CTD) of RNA polymerase II (Wallenfang and Seydoux, 2002).

Becoming singletons on CDK7 and cyclin H genes may be common tendencies in a wide variety of organisms with a polyploidy genome. Cyclin H gene becomes a singleton in the genome of zebrafish, medaka and *Arabidopsis thaliana* (Wang et al., 2004), which underwent whole genome duplication 300 (teleosts) and 30–40 Mya, respectively. The CDK7 gene becomes a singleton together with some other CDKs in the genomes of medaka and zebrafish (data not shown), and plants' genes coding for CDK7 are conserved with low copy numbers after repeated whole genome duplication (Lehti-Shiu and Shiu, 2012). Since CDK7 and cyclin H construct CAK, the activity of which is essential for all CDKs' activation and acceleration of cell cycling, a loss of control in their expression leads to serious results such as embryonic lethality (Larochelle et al., 1998). Singleton or low copy number genes may have simplified their regulation compared to maintaining paired or high copy number genes.

CDKIs are generally retained at seven genes in many animals' genomes, and their loss-of-function mutations lead to severe diseases including cancers (Harper and Elledge, 1996; Roussel, 1999; Martin, 2009). However, in the *Xenopus* genome, we found that the genes coding for CDKns are very fragile: the *X. laevis* genome has no gene for CDKn1c and CDKn2a, and the *X. tropicalis* genome has an additional gene loss for CDKn1b (Table 2). In humans, such loss-of-function mutations often result in Beckwith-Wiedemann syndrome, which is characterized by macrosomia, macroglossia, tumor predisposition and congenital malformations (CDKn1c, Weksberg et al., 2005; Besson et al., 2008; Romanelli et al., 2010), and many kinds of cancers (CDKn1b, Ruan and Peters, 1998;

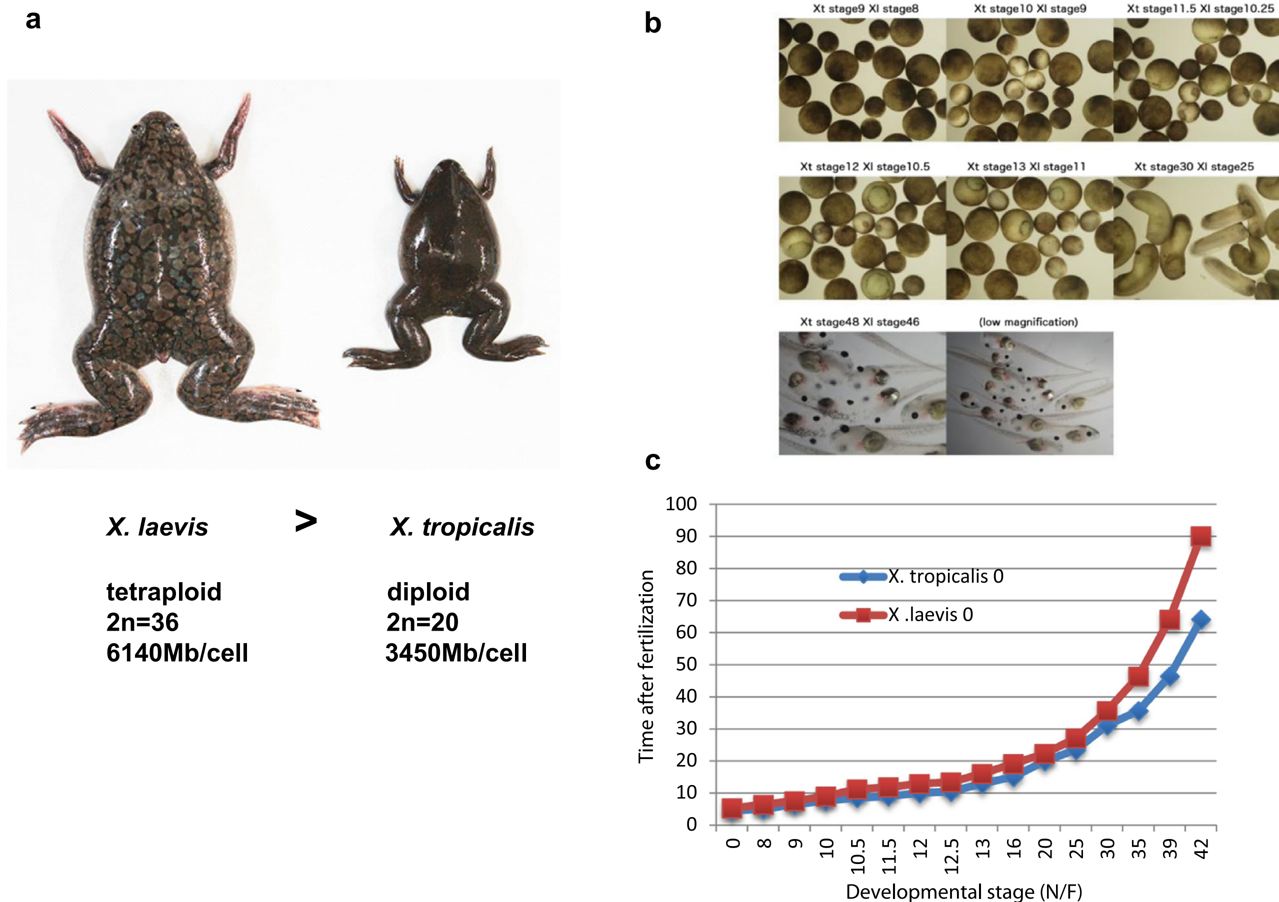

Fig. 7. Comparative analysis of developmental speed between *X. laevis* and *X. tropicalis*. (a) Appearances of *X. laevis* and *X. tropicalis*. (b) Appearances of developing embryos. The stages of *X. laevis* and *X. tropicalis* at each time point are described above the photo. (c) Growth of *X. laevis* and *X. tropicalis* is summarized. After fertilization, eggs of *X. laevis* and *X. tropicalis* were simultaneously cultured at 26 °C, and times were determined at the indicated stages (b and c).

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Roussel, 1999; Besson et al., 2008), respectively. In particular, CDKn2a is known as a representative tumor suppressor, and loss of its function increases the risk of a wide variety of human cancers (Ruan and Peters, 1998). In addition, the gene locus for CDKn2a encodes another gene product, p14ARF, in the human genome (Quelle et al., 1995). The transcript of *arf* contains a different first exon from CDKn2a, which is located upstream of the first exons for CDKn2a, and a common second exon to CDKn2a, which is translated via two different reading frames. While the two transcripts with different first exons, each of which includes initial Met, produce two distinct proteins, both proteins have a common role to suppress cancers with different mechanisms (Sherr, 1998; Sharpless and DePinho, 1999). Since the *Xenopus* genome has no locus for CDKn2a, it lacks not only CDKn2a but also ARF. We also found that one of the homeologous gene pairs of Rb, which is a representative tumor suppressor gene in humans, is lost from the L-chromosome (data not shown). Since it was reported that cancers are not frequent during the long lifespans of *Xenopus* compared to mammalian models (Ruben et al., 2007; Hardwick and Philpott, 2015), they may have other mechanisms for suppressing cancers. Although at least the immune system and apoptosis system seem to be concerned with the resistance to tumors in *Xenopus* (Ruben et al., 2007; Hardwick and Philpott, 2015), it is also possible that the novel CDKI gene, which specifically appeared on the *Xenopus* genome, and low expression of CDK7/cyclin H play this role. The alterations of CDKIs genes are caused by the fragility of genes in *Xenopus*, but this property may result in other mechanisms to suppress cancers in *Xenopus*. Detailed analysis is still required.

Since the alterations of genes coding for CDKn1c, CDKn2a and CDKnx are included in the genomes of both *Xenopus laevis* and *tropicalis* (Table 2), the alterations occurred before the speciation of *X. laevis* and *X. tropicalis*, 48 Mya (Session et al., 2016). In addition, comparative analysis shows that the coding sequences for CDKnx are different among *X. tropicalis* and chromosomes L and S of *X. laevis* (Fig. 2), implying that these alterations occurred indepedently during relatively short periods after the speciation of *X. laevis* and *X. tropicalis*, 48 Mya(Session et al., 2016). Although *X. tropicalis* has a deletion of CDKn1b gene on its genome, *X. laevis* has the genes as homeologues, indicating that the deletion of CDKn1b from the *X. tropicalis* genome also occurred within 48 million years. As for CDKn2c, which is retained as homeologous genes in *X. laevis*, one of them has substitutions of amino acids, leading to a severe decrease of its function (Fig. 5). Not only the coding sequence of CDKIs, but also their regulatory region can be changed between chromosomes L and S (Fig. 4). These data totally indicate that the genes coding for CDKIs are very fragile and changeable in *X. laevis*, though these genes are generally stable in a wide variety of animals (Table 2) because of their roles in the development and health of animals (Sherr and Roberts, 1995; Sherr and Roberts, 1999; Roussel, 1999; Kato et al., 2001). On the other hand, the fragilities of some kinds of important genes in allotetraploidy might allow occurrences of novel genes, which have novel functions and novel expression patterns, in evolution.

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