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

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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 deaccelerates cell-cycling in Xenopus.
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 CDK1c and CDK1a2 are lost in both homeologous loci, and a gene classified into the CDK1 family novelly appears. Also, another gene coding CDK1b 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 TrueSeq 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 + JGL_gene_v1_4_Scaffold53263_1780616.1797615, cdkn2c_S-DNA + Clones + JGL_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).

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 17–18 Mya (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.

Primer linker sequences are shown in lowercase letters. The locations of the cis-regulatory elements in the genome assemblies are in Chr04:85960521–85960766 (*Xtropicalis* ver. 9) and chr4:69927237–69927451 (*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.
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 genes coding for cyclin-dependent kinase inhibitors are fragile in Xenopus.
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 CDKIs included in CDK1 and CDK2 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 CDK1c (p57kip2) and CDK2a (p16ink4a) are deleted from both chromosomes L and S. Instead, a novel CDKI gene, which is classified into the CDK1 family, appeared in chromosomes 7L and 7S (Table 2). These alterations of CDKI genes are common in Xenopus and dogs (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 CDK1b (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,
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 CDKnx.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 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. 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. 2.** Comparisons of amino acid sequences of CDKnx (Xic1) among X. tropicalis (Xtr) and chromosomes L (XIL) and S (XIS) of X. laevis. Alignments were calculated from data based on the gene models (Xtropicalis 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.

**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.
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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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

**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.
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 CDKn1s 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; CDKn2a, Harper et al., 1998).

![Fig. 6. Synteny 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.](image)

**Fig. 6.** Synteny 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.

**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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<tr>
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Rousseau, 1999; Besson et al., 2008), respectively. In particular, CDKnis are 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 loci for CDKn1a encodes another gene product, p14ARF, in the human genome (Quelle et al., 1995). The transcript of afi contains a different first exon from CDKn2a, which is located upstream of the first exons for CDKn1a, 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). It was reported that cancers are not frequent during the long lifespan 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 CDK1 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 CDK1c, 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 independently 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 homologues 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; Rousseau, 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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