Int. J. Dev. Biol. 53: 215-229 (2009)
doi: 10.1387/ijdb.082644jg

THE INTERNATIONAL JOURNAL OF DEVELOPMENTAL BIOLOGY
www.intjdevbiol.com

Dynamic alterations of linker histone variants during development

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ABSTRACT The process of development can be viewed as a series of linker histone replacements which take place throughout spermatogenesis and oogenesis, as well as following fertilization or somatic nuclear transfer (SNT). Although few of the histone H1 variants in question have been shown to be essential for viability, the timing of their appearance as well as the affinity with which they are able to bind to chromatin seem to be important factors in their developmental role. A looser binding of linker histones to chromatin seems to correlate with the meiotic phases of gametogenesis and the establishment of a totipotent, as well as the maintenance of a pluripotent, state in early embryos, while tighter binding of linker histones to chromatin appears to be associated with the mitotic phases, as well as the increased levels of condensation that are required for the packaging of DNA into sperm. This latter process also involves the binding of certain basic non-histone proteins to DNA. While all proteins involved in chromatin compaction during development are highly basic in nature, in general they can be seen to change from lysine-rich variants to arginine-rich ones, and back again. The fact that linker histone transitions are conserved across diverse metazoan species speaks of their importance in packaging DNA in a variety of ways during this crucial period.

KEY WORDS: *histone H1, spermatogenesis, oogenesis, fertilization, SNT*

Chromatin structure

The DNA in our cells is in a constant state of flux. Not only must the approximately two meter long length of DNA in human cells be packaged tightly in order to transmit it to other cells, it must also be periodically unpackaged in order to use any of the information stored in its code, such as during the processes of transcription and DNA replication. DNA is normally packaged in the cell in the form of chromatin. Chromatin consists of DNA wrapped around an octamer of the four core histone proteins: H2A, H2B, H3, and H4, to form a nucleosome. This arrangement was first described in 1974 by now Nobel Laureate Roger Kornberg, a finding that he recently reviewed and reflected upon (Kornberg, 1974; Kornberg and Lorch, 1999). One year later, the name «nucleosome» was coined to describe this fundamental unit of chromatin (Oudet *el al.*, 1975). An open conformation of chromatin that is amenable to transcription or replication is commonly pictured as a string of nucleosomes linked together by connecting «linker» DNA, also known as a «beads on a string» arrangement, or simply a 10 nm fiber. The addition of a fifth «linker» histone, histone H1, which interacts with this connecting DNA was long pictured to be responsible for the conversion of this open arrangement to a closed one which is more refractory to cellular processes, a 30 nm fiber whose formation was dependent upon this final histone binding, although it has also been seen that this structure is further stabilized by a number of the core histone «tails» (Schwarz *et al.*, 1996). These tails are features of the tripartite structure which makes up all histones, which contain a central globular domain surrounded by these N- and C-terminal extensions. The 30 nm fiber is then thought to go through a hierarchical series of «higher order» folding before it achieves the final folded state of the metaphase chromosome.

Histones are very basic proteins, owing to the large numbers of lysine and arginine residues which are primarily associated

Abbreviations used in this paper: ES, embryonic stem; FRAP, fluorescence recovery after photobleaching; MBT, mid-blastula transition; MMTV, mouse mammary tumor virus; PGC, primordial germ cells; SNT, somatic nuclear transfer.

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Published online: 2 February 2009.

ISSN: Online 1696-3547, Print 0214-6282
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with their tails (Ramakrishnan, 1997). Covalent chemical modification of these, as well as other amino acids, both in the tails and in the globular domain of histones is thought to modulate their binding to DNA as well as their compaction into chromatin, a concept referred to as the «histone code» (Strahl and Allis, 2000). Chemical modification of the histones, however, is not the only means that the cell uses to modulate their interaction with chromatin. All of the histones, with the exception of histone H4, are known to exist in structural variants which differ from one another in their primary sequence; these variants are often differentially expressed (Ausio, 2006). The number of linker histones variants, as well as their degree of divergence from one another, is much greater compared to the core histones (Brown, 2001). There are no less than eleven linker histone variants that have been found associated with mammalian cells, this is more than twice the number of variants found of any core histone (Ausio, 2006). The variants can be broadly classified as those commonly found in somatic cells (histones H1.0-H1.5, as well as H1.X) and those found associated with a particular tissue type. The somatic histones are also referred to as histones H1°, H1a, H1c, H1d, H1e, H1b, and H1x, respectively, for a full discussion of linker histone nomenclature, see our recent review (Godde and Ura, 2008). Tissue-specific linker histones have been found both in the testes during spermatogenesis (H1t, H1t2, and HILS) as well as in the oocyte during oogenesis (H1oo) (Ausio, 2006). In addition to their classification according to the cell type that they are expressed in, linker histone variants are also commonly classified as being either DNA replication dependent or independent in their expression patterns (Ausio, 2006). Most of the somatic histones are in this former class, being expressed only in S-phase, with the exception of H1° and H1x, which are expressed primarily during G_0 and G_1 phases, respectively. The tissue specific variants of H1 also fit into this latter class, since their expression is also replication independent. This class of linker histones has also been referred to as «replacement» histones, since they serve to replace the replication dependent ones during various times in the cell cycle (Ausio, 2006). The time where histone replacement is most evident, as we will discuss, is during the process of development, which can be viewed in terms of the linker histone replacements which are taking place during specific stages (for an early review, see Khochbin and Wolffe, 1994). We will return to this discussion after we have expanded upon the *in vivo* function of histone H1 and its variants.

Function of linker histones

A wealth of *in vitro* data has demonstrated that histone H1 binds to the outside of the nucleosome to form a «chromatosome», in which it protects an additional 20 bp of DNA from cleavage by nucleases, compared to the 146 bp of DNA which is wrapped around the octamer of core histones, in a process which normally serves as a prerequisite for the formation of the 30 nm fiber (Kornberg and Lorch, 1999). Linker histone binding was thus pictured as a means to achieve a general repression of cellular processes such as transcription, in view of its ability to block the access of the cellular machinery to folded chromatin. It thereby came as somewhat of a shock in 1995 when the H1 gene was knocked out in the ciliated protozoan *Tetrahymena thermophila* without an effect on cell viability or growth (Shen *et al.*, 1995). This finding was then followed over the next decade by an expanding list of organisms that could live without histone H1, the deletion of which had only minimal effects. Since we have recently reviewed these studies in detail, we will not attempt to rehash all of the pertinent H1 deletion studies here, and the reader is referred to our previous work (Godde and Ura, 2008). We will, however, reexamine some of these studies in terms of their relation to the developmental process. While the deletion of linker histones has been shown to be without an effect on the viability of a number of organisms, the existence of some role for them in the developmental process remains unquestionable. Certain clues to their precise role during development have been brought to light by the genetic studies to be described below.

A number of lower eukaryotes which lack complex developmental processes have been found to survive in the absence of histone H1 with few discernable effects. This includes *Tetrahymena*, along with three fungal species: *Saccharomyces cerevisiae*, *Aspergillus nidulans*, and *Ascobolus immersus* (Ushinshy *et al.*, 1997; Ramon *et al.*, 2000; Barra *et al.*, 2000). The latter species is of most interest to this discussion, since the authors did note that H1 deletion led to a hypermethylation of DNA at specific sites, primarily those which were found to have some degree of methylation in the wild-type strains, a link that has also been observed in higher eukaryotes (Barra *et al.*, 2000). H1 deletion studies have proved to be more difficult in higher eukaryotes, however, due to the presence of multiple H1 subtypes encoded by separate genes, along with a little-understood compensatory mechanism which up-regulates the expression of remaining subtypes so that near normal ratios of linker histones to nucleosomes are maintained (Sirotkin *et al.*, 1995). This compensatory mechanism, although first described in animals, is also present in plants, as was seen when the levels of two major histone H1 variants were decreased in transgenic tobacco plants (Prymakkowska-Bosak *et al.*, 1999). In these studies, the H1-depleted plants were shown to exhibit male sterility, along with aberrations in flower development which could be linked to changes in the temporal expression of specific genes. The link between histone H1 and the maintenance of specific methylation patterns in DNA was further supported by studies from this same group, which were subsequently able to achieve a >90% reduction in linker histone levels in *Arabidopsis thaliana* (Wierzbicki and Jerzmanowski, 2005). These mutants displayed a number of developmental aberrations, including the formation of irregular leaves and changes in both the timing and morphology of flower production, which were reminiscent of *Arabidopsis* plants known to display DNA hypomethylation in specific regions. As expected, the authors were able to demonstrate altered patterns of DNA methylation in the H1 mutant strains (Wierzbicki and Jerzmanowski, 2005).

A majority of the genetic studies demonstrating the importance of histone H1 in animals have been performed in mice, many of which have been undertaken by the laboratory of Arthur Skoultchi. Initial studies by this group determined that single gene knockouts of various H1 variants produced no detectable effects, probably due to the aforementioned compensatory mechanism, demonstrating in this case that neither H1° nor H1t were indispensable in the mouse (Sirotkin *et al.*, 1995; Lin *et al.*, 2000). Two subsequent studies by different laboratories confirmed this latter finding concerning the dispensability of histone H1t (Drabent *et al.*, 2000; Fantz *et al.*, 2001). A review of histone H1 diversity written

around this time put forth a theory that the timing of histone variant expression may, in fact, be more important than their actual primary sequence (Khochbin, 2001). This would account for the compensatory mechanism's ability to replace a specific H1 subtype by the up-regulation of a similar gene. This mechanism was therefore put to the test by the deletion of two genes simultaneously: knocking out H1°, along with either H1.2, H1.3, or H1.4, however, still did not result in a change in the ratio of linker histone to nucleosomes in the chromatin of the double mutants and was still without discernable effect (Fan *et al.*, 2001). It was only when H1t, along with the histone that was found to be mainly responsible for its replacement in single mutants, histone H1.1, was deleted that a 25% reduction in the normal ratio of H1 to nucleosomes was achieved and that some subtle effects in the gene expression of mutant cells could be demonstrated (Lin *et al.*, 2004). It was not until triple mutants (H1.2, H1.3, and H1.4) were created in mice that the normal ratio of H1 to nucleosomes was reduced by 50%, resulting in severe developmental defects (Fan *et al.*, 2003). Embryos which carried this triple mutation usually died by midgestation, exhibiting a broad range of defects. A follow-up paper described the monitoring of changes in gene expression which may have led to the observed defects in these mutants using microarrays (Fan *et al.*, 2005). Here, it was shown that 29 genes in mice exhibited at least a 2-fold difference in expression between mutants and the wild type, among which the authors noticed an overrepresentation of imprinted genes, as well as those associated with the X-chromosome. In all, nearly one third of the affected genes were known to be normally regulated by changes in DNA methylation (Fan *et al.*, 2005). Thus, the links between histone H1 and DNA methylation have been demonstrated across three kingdoms: fungi, plants, and animals (Barra *et al.*, 2000; Wierzbicki and Jerzmanowski, 2005; Fan *et al.*, 2005). Since these links, as well as the phenotypes of both plants and animals with severe reductions in their level of linker histones, all point to a central role for histone H1 and its variants during development, we will next describe the various developmental stages, along with the corresponding changes in H1 subtype which accompanies them.

Spermatogenesis

Spermatogenesis is the process by which spermatogonial stem cells go through a series of morphological changes in order to produce the differentiated haploid cells known as spermatozoa (Pradeepa and Rao, 2007). The process can be divided into three stages: 1) the mitotic, or proliferative, phase, in which spermatogonial stem cells undergo multiplication and renewal, 2) the meiotic phase, in which the genetic material of spermatocytes is recombined and segregated, and 3) the differentiation, or spermiogenic, phase, in which spermatids elongate and transform into mature spermatozoa (Russell *et al.*, 1990; Pradeepa and Rao, 2007). We will discuss the linker histone complement of each of these stages in turn.

Mitotic phase

Primordial germ cells, or PGCs, that migrate to the developing male testis become arrested in the G_1 phase of the cell cycle until the birth of the organism. These spermatogonial stem cells must then undergo a number of mitoses as they differentiate to form spermatocytes. In the rat, spermatogonia divide approximately nine times a week (Russell *et al.*, 1990). Spermatogonia are further classified into either Type A, Intermediate, or Type B, depending on the levels of chromatin that can be found lying along the inner aspect of the nuclear envelope using microscopy, exhibiting either none, a moderate amount, or a large amount, respectively (Russell *et al.*, 1990). Spermatogonia have been found to contain a compliment of somatic linker histones, namely H1.1 and H1.2 (Meistrich *et al.*, 1985; Fig. 1). Knocking out the H1.1 gene, in agreement with the studies described above, did not have an effect on spermatogenesis (Rabini *et al.*, 2000). Instead, it was shown that levels of H1.2 increased in the mutants, along with that of H1.3 and/or H1.4. Interestingly, RNA-mediated interference of H1.1 in *Caenorhabditis elegans* affected germline proliferation and differentiation, often leading to mutant sterility, but this was true of hermaphrodites only, not of males (Jedrusik and Schulze, 2001). This was not, however, the case when the expression of one of the other five somatic H1 subtypes was disrupted.

A number of *in vitro* studies have been performed to compare the ability of different histone H1 subtypes to condense short arrays of nucleosomes (De Lucia *et al.*, 1994; Khadake and Rao, 1995; Talasz *et al.*, 1998). One of these studies used circular dichroism to gauge the effect of linker histone binding to H1-depleted chromatin and concluded that histone H1.1 had the highest condensation ability of all the subtypes tested (De Lucia *et al.*, 1994). Another study used similar methods to conclude that the

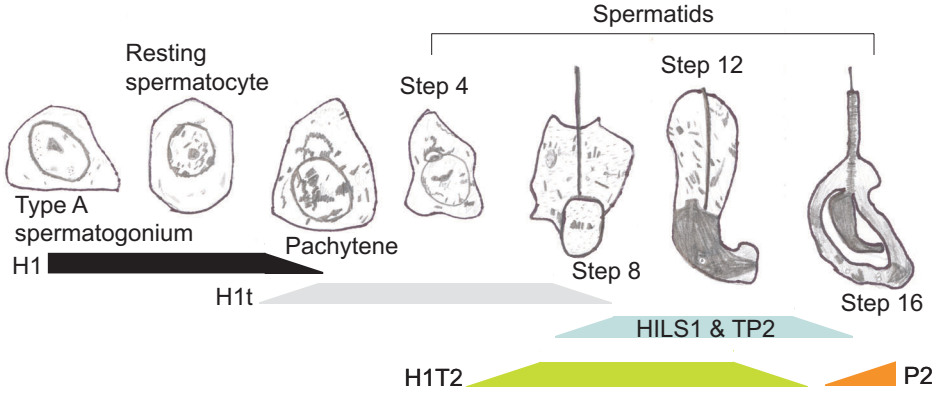

Fig. 1. Morphological changes during spermatogenesis, along with known linker histone transitions. *An overview of the major developmental changes which occur during the formation of sperm in mice. For simplicity, not all stages are shown. Width of bar corresponding to a particular H1 subtype or non-histone variant is approximate for the amount of each protein that has been detected in the various stages shown. Histone and non-histone variants are labeled; somatic H1 levels are indicated by a black bar, H1t levels by a gray bar, H1T2 levels by a green bar, HILS1 as well as TP2 levels by a blue bar, and P2 levels by an orange bar. Levels of protein are only approximate, based on sources which are referenced in the text. Illustration by Justin Godde.*

ability of histone H1.1 to condense chromatin was roughly the same as a mixture of subtypes purified from rat liver (Khadake and Rao, 1995). A third experiment studied the ability of increasing amounts of histone subtypes to aggregate chromatin arrays which had been reconstituted *in vitro* onto a specific DNA sequence and confirmed that, while the chromatin condensing activities of H1.1 were roughly equivalent to a mixture of H1 subtypes from liver tissue, they were higher than that of a mixture of subtypes obtained from testes, as well as of purified histones H1.2, H1.5, and H1t (Talasz *et al.*, 1998). Since mitotic chromosomes represent the most condensed state in the cell that DNA can obtain, it is not surprising that histone H1 subtypes known for their chromatin condensation abilities predominate during the mitotic phase of spermatogenesis. At the end of the mitotic phase, Type B cells divide to form preleptotene, or resting, spermatocytes (Russell *et al.*, 1990; Fig. 1).

Meiotic phase

Resting spermatocytes can be microscopically differentiated from Type B spermatogonia by observing their chromatin. The former appear slightly smaller than the latter, having less chromatin situated along the nuclear envelope, as well as less protruding inward toward the center of the nucleus (Russell *et al.*, 1990). These cells will then undergo two meiotic divisions, the first of which forms secondary spermatocytes and the second of which produces spermatids. Meiosis I is characterized by an exceptionally long prophase, lasting about three weeks in the rat, the five stages of which can been discriminated from each other by the appearance of the chromatin (Russell *et al.*, 1990). In leptotene cells, the nuclei lose their peripheral chromatin and form condensed meiotic chromosomes, which are visible as fine chromatin threads (Russell *et al.*, 1990). The chromosomes then pair along their length in zygotene cells, and remain paired for an extended pachytene stage that lasts for nearly two weeks in the rat (Russell *et al.*, 1990). It is during this stage that homologous recombination, or crossing over, takes place, while the spermatocyte cells can be seen to rapidly increase in size as their enlarged nuclei change in appearance from round to ovoid in shape (Russell *et al.*, 1990). It is midway through pachytene that the linker histone type undergoes its first replacement in spermatogenesis (Fig. 1).

The presence of testis-specific linker histones was first reported over 30 years ago. In 1975, the same year the nucleosome got its name, frog testes were reported to contain a fast migrating H1 variant that was not found in other tissues (Alder and Gorovsky, 1975). The same year, this finding was expanded to rat, rabbit, monkey, and mouse (Shires *et al.*, 1975). Within five years, this protein had been isolated from rat and its amino acid content analyzed. The isolated variant was named histone H1t, and its relationship to meiosis was noted, along with its characteristic enrichment in arginine (Seyedin and Kistler, 1980). Linker histones, being quite basic proteins, normally contain lysine as their most prevalent amino acid. While lysine had been found to be present at almost a twenty fold higher amount than arginine in other H1 variants, it was found to be only three fold enriched in H1t (Seyedin and Kistler, 1980; Table 1). When the amino acid sequence of histone H1t was determined (in this case from boar), it was found that most of the replacement of lysine with arginine occurred in the C-terminal tail, which was also seen to be shorter than the comparable domain of somatic H1s (Cole *et al.*, 1984; Fig. 2). As we shall see, the process of linker histone replacement which takes place during spermatogenesis could also be characterized as a progressive increase in arginine-rich proteins which is accompanied by the steady decline, and eventual disappearance, of the lysine rich ones (Table 1; Fig. 1).

A wealth of collected evidence suggests that histone H1t both binds to DNA with a lower affinity than somatic H1 as well as condenses chromatin to a lesser extent upon binding (De Lucia *et al.*, 1994; Khadake and Rao, 1995; Talasz *et al.*, 1998; Wellman *et al.*, 1999; De *et al.*, 2002; Ramesh *et al.*, 2006). Significantly, certain lysine rich repeats, (S/T)P(K/A)K, that are known for their interaction with DNA as well as for acting as sites of phosphorylation associated with the formation of condensed chromatin, were found to be absent in histone H1t (Cole *et al.*, 1984). The three *in vitro* studies discussed above in the context of H1.1 binding to chromatin arrays all agreed that H1t was a poor condenser of chromatin, relative to the somatic H1 subtypes (De Lucia *et al.*, 1994; Khadake and Rao, 1995; Talasz *et al.*, 1998). Furthermore, when the affinity of histone H1t for naked DNA was compared to that of H1°, it was seen that the former occupied a smaller binding site as well as exhibited binding constants which were one to two orders of magnitude lower than the latter histone (Wellman *et al.*, 1999). Ever since the amino acid sequence of H1t was first determined, it was suggested that the C-terminal tail would prove to be the most critical determiner of this histone's interaction with the nucleosome. Susan Wellman, from the above referenced article soon went on to collaborate with another research group at her institution to create recombinant chimeric histones which contained various combinations of the three histone domains, taken from either H1t or H1°. Investigations of chromatin folding using fluorescence microscopy, as well as functional studies of the inhibition to DNA replication afforded by the various constructs both showed that the C-terminal tail region was the domain which was responsible for the different properties exhibited by these two histones (De *et al.*, 2002). The C-terminal histone tail, however, may not be the only determinant of reduced H1t binding to chromatin. Recently, Satyanarayana Rao's group, which is referenced above, showed that a single amino acid change in the globular domain of histone H1t can increase its affinity for nucleosomes to comparable levels with histone H1.3, the two histones normally differing in globular domain sequence at this single location (Ramesh *et al.*, 2006).

All of these *in vitro* studies point to histone H1t forming less compact chromatin with a more open arrangement when it is

TABLE 1

CHARACTERISTICS OF LINKER HISTONES AND THE RESULT OF THEIR DEPLETION IN MICE

H1 variant	Size (a.a.)	K:R	Depletion Result	Method	Reference
somatic*	212-223	16	Developmental defects	K.O. (triple)	Fan *et al.*, 2003
H1t	209	2.9	None detected	K.O.	Lin *et al.*, 2000
H1T2	398	0.6	Reduced fertility	K.O.	Martianov *et al.*, 2005
HILS1	170	1.6	Reduced sperm mobility?	n.d.	Jedrzejczak *et al.*, 2007
TP2	117	0.7	Smaller litters	K.O.	Zhao *et al.*, 2001
P2	107	0.08	Heterozygote is sterile	K.O.	Cho *et al.*, 2001
H1oo	304	2.3	Metaphase I arrest	antisense MO	Furuya *et al.*, 2007

Particular H1 variants discussed in this review, along with their size, lysine to arginine ratio (K:R), and result of depletion in mice. Methods of depletion include: K.O. (genetic knockout), Antisense MO (morpholino oligonucleotides), or n.d. (not done). TP2 and P2 are included in this chart, even though they are not classified as linker histones. *somatic H1 denotes averaging of data from H1.1-H1.5, the triple knockout referred to is that of H1.2-H1.4 only.

bound. Such a chromatin arrangement might be particularly amenable to the genetic recombination which is known to take place during this stage of spermatogenesis. This is not to say that H1t is the sole linker histone variant found during the meiotic phase. In prepachytene spermatocytes, histone H1.1 has been estimated to comprise about 70% of the linker histone in the cell (Lennox and Cohen, 1984). These levels are then seen to steadily decrease at the beginning of this phase, although the histone does not disappear entirely until the middle of the post meiotic phase (Chuirkov *et al.*, 2004). Levels of H1t, on the other hand, are seen to rapidly increase during the pachytene stage until it becomes the predominant linker histone (Meistrich *et al.*, 1985). Two of the studies which knocked out histone H1t in mice measured the amount of both H1.1 and H1.2 in mutant testes and found that levels of the two had risen from about 30-40% of the H1 compliment to approximately 40-50% for the former and from around 15-20% to about 30% for the latter (Lin *et al.*, 2000; Drabent *et al.*, 2000). The authors went on to surmise that the depletion of H1t may be tolerated in spermatocyte chromatin due to the maintenance of this less compact chromatin arrangement in the mutants (Drabent *et al.*, 2000). It was not investigated whether the H1t mutants had defects in genetic recombination, something that would not be expected to produce an obvious phenotype but could, instead, ultimately have long-term effects on the genetic diversity of a given population.

Following the long pachytene stage, the next two stages of prophase I occur fairly rapidly. First, the diplotene stage is characterized by the separation of the chromosome pairs, except at chiasmata; then, during diakinesis, the chromosomes become even more condensed and the chiasmata are forced to move laterally, toward the tips of the chromosomes (Russell *et al.*, 1990). Similarly, metaphase, anaphase, and telophase of meiosis I are completed quite rapidly as well, the first meiotic division culminating with the formation of secondary spermatocytes. These spematocytes are similar in appearance to the primary spermatocytes from which they arose, with the exception of being 30-40% larger and containing heterochromatin which appears somewhat fuzzy, as opposed to being fairly distinct (Russell *et al.*, 1990). Meiosis II then takes place, again quite rapidly, to produce haploid spermatids from the secondary spermatocytes.

Differentiation phase

In the rat, three more weeks are required for spermatids to differentiate into mature spermatozoa, a process which does not

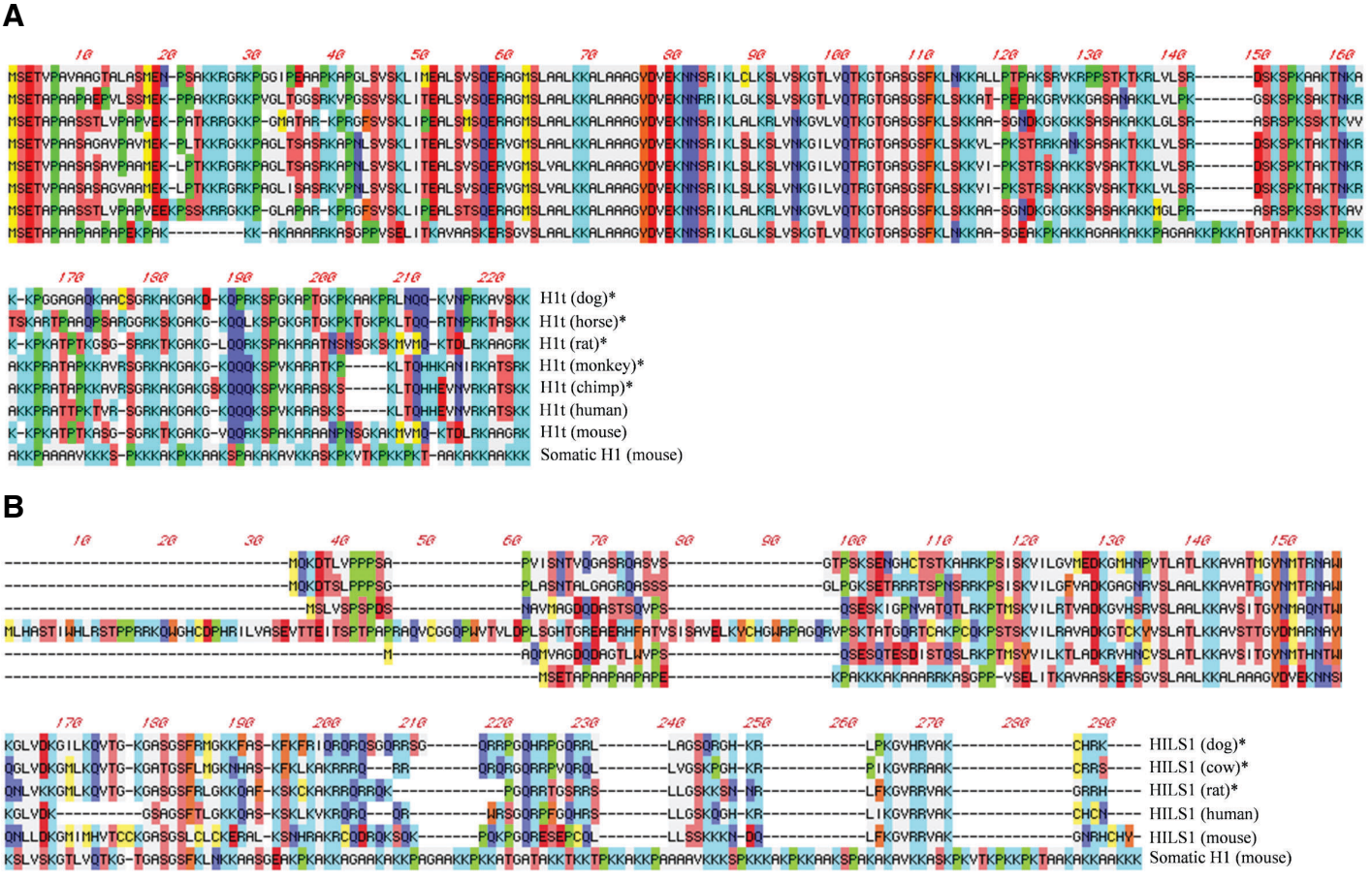

Fig. 2. Multiple alignment of protein sequences from histone H1t and HILS1. *Protein sequences from a variety of species aligned using a ClustalW alignment. Sequences from either* **(A)** *H1t or* **(B)** *HILS were aligned to a consensus sequence formed from somatic H1.1-H1.5. An asterisk denotes hypothetical proteins whose assignments are tentative. Alignment was performed using MacVector 7.2.2 program using default ClustalW settings. The accession numbers of the protein sequences used are, from top to bottom: (A) XP_545388, XP_001497342, NP_036711, NP_001074230, XP_527257, NP_005314, NP_034506; (B) XP_537672, XP_873430, NP_001103035, AAQ23050, NP_061262.*

require any further cell division but is accompanied by a burst of transcription (Russell *et al.*, 1990). This spermiogenic process is further subdivided into 19 steps in the rat but only 16 in the mouse, in which round spermatids undergo a number of morphological steps to become elongated spermatids (Russell *et al.*, 1990). While the cell transformations which occur during this phase include the development of both the flagellum as well as the acrosome (a golgi-derived sac of enzymes found in the sperm head), in addition to the elongation and significant elimination of the cytoplasm, this review will continue to concern itself primarily with the changes which take place in chromatin structure as well as linker histone type. During the elongation of round spermatids, it was recently shown that another histone H1 subtype, aptly called H1T2, appears and gradually replaces H1t (Martianov *et al.*, 2005; Tanaka, H., *et al.*, 2005; Figs. 1 & 3). H1T2 is distinctive in that it is even more rich in arginine that H1t, with almost twice as many arginine residues as lysines, and, in contrast to H1t, there are 14 potential phosphorylation sites (Tanaka, H., *et al.*, 2005; Table 1). Phylogenetic analysis showed that this histone is more closely related to histone H1° than it is to H1t (Tanaka, H., *et al.*, 2005). H1T2 expression is first detected in step 4 of differentiation, after round spermatids have begun to take on an ovoid shape in preparation for elongation (Martianov *et al.*, 2005; Russell *et al.*, 1990; Fig. 1). This linker histone specifically localizes to chromatin in the apical pole just beneath the developing acrosome, opposite from where the flagella will begin to sprout around step 8. Expression continues in the spermatid until step 13, when the cell has obtained an elongated shape, but the cytoplasm remains somewhat ovoid in shape, prior to undergoing significant elimination (Tanaka, H., *et al.*, 2005; Russell *et al.*, 1990; Fig. 1).

Deletion of the H1T2 gene was found to lead to severely reduced fertility in males, accompanied by morphological abnormalities such as residual cytoplasm, acrosome detachment, and fragmented DNA (Martianov *et al.*, 2005). These morphological abnormalities were severe enough to prevent fertilization, both *in vivo* and *in vitro*, but mutant sperm remained capable of fertilizing eggs by intracytoplasmic injection (H. Tanaka *et al.*, 2005). Subsequent studies investigated the mechanisms that led to the polar placement of H1T2-rich chromatin and found that it was not dependent on the formation of the acrosome itself but was sensitive to mutations in proteins known to play a role in the organization of nuclear chromatin (Catena *et al.*, 2006). In these mutants, the H1T2 localized in a bipolar fashion, preventing proper elongation of the spermatids.

In step 9, shortly after the flagella become visible, a third testis-specific H1 subtype, HILS1 (H1-like protein in spermatids 1), whose expression overlaps with that of H1T2, is seen to be expressed in elongating spermatids (Yan *et al.*, 2003; Iguchi *et al.*, 2004; Fig. 1). HILS1 was found to have a lysine to arginine ratio which was intermediate between the two other sperm-specific linker histones, and phylogenetic analysis of the HILS1 gene showed that, unlike H1T2, it is closely related to H1t (Table 1; Fig. 2; Yan *et al.*, 2003). The appearance of HILS1 coincides with the disappearance of histone H1t, in addition to that of the core histones which have been present during spermatogenesis (Yan

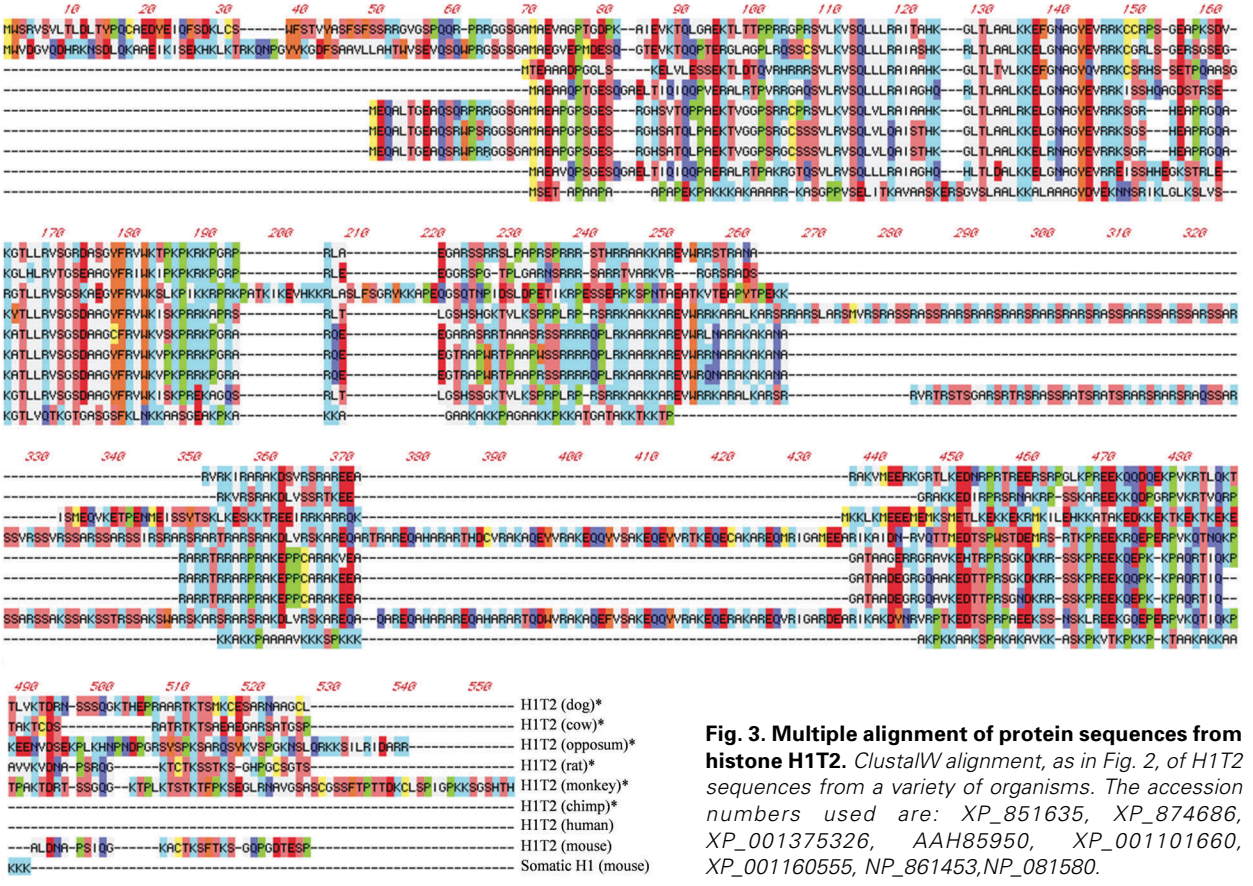

Fig. 3. Multiple alignment of protein sequences from histone H1T2. *ClustalW alignment, as in Fig. 2, of H1T2 sequences from a variety of organisms. The accession numbers used are: XP_851635, XP_874686, XP_001375326, AAH85950, XP_001101660, XP_001160555, NP_861453,NP_081580.*

et al., 2003). Although HILS1 has been shown to bind to reconstituted nucleosomes *in vitro*, is does so with a lower affinity, compared to histone H1.1 (Yan *et al.*, 2003). Despite this, its lack of significant overlap with core histones during development suggests that HILS1 has the distinction of being the only linker histone that does not normally coexist with nucleosomes. Although the deletion of HILS1 has not been reported in mammals, a disruption of Mst77F, a purported homolog in *Drosophila melanogaster*, speaks to its importance in spermatogenesis (Raja and Renkawitz-Pohl, 2005). Mst77F mutant males are sterile and can be seen to retain round nuclei in their spermatids, instead of elongated ones. In addition, the levels of HILS1 have been found to be significantly reduced in human males suffering from reduced sperm motility (Jedrzejczak *et al.*, 2007).

What form does DNA take at this point, if not found complexed into canonical nucleosomes? Around the time H1t and the core histones disappear, a set of small basic proteins, including transition protein 1 (TP1 of TNP1) and transition protein 2 (TP2 or TNP2), appear in the spermatids (Oko *et al.*, 1996, Fig. 1). These proteins are seen to be quite arginine rich, with ratios between the two basic amino acids comparable to that of H1T2 (Table 1; Yu *et al.*, 2000). TP2 is 13 kDa in size, approximately the size of one of the core histones, while TP1 is less than half that molecular weight. It has been suggested that the latter is involved in histone removal, while the former is involved in chromatin condensation (Zhao *et al.*, 2001). In fact, TP2 can be seen to co-localize in the cell with HILS1 at this point (Yan *et al.*, 2003). The transition proteins begin the alteration between the chromatin that has existed up until this point and a highly condensed, uniformly dense mass that forms beneath the acrosome. Upon the appearance of the transition proteins, transcription in the spermatid can be seen to cease and chromatin takes the form of a smooth fiber (Oko *et al.*, 1996). The DNA will eventually be complexed into its most dense form that is found associated with any cell and be packaged into a volume that is less than 5% that of a somatic cell nucleus, in preparation for its transport to the oocyte (Cho *et al.*, 2001). By step 13 of the differentiation phase, when transcription of H1T2 has been turned off, and the protein can be seen to disappear from the cell, HILS1 is, presumably, the only linker histone which remains in the cell, albeit at relatively low levels at this point (Fig. 1). Basic proteins that can be extracted from the nucleus at this stage have been estimated to consist of 62% TP1, 28% TP2, and only 1% histone (Yu *et al.*, 2000).

Transition proteins are not only similar to histones in their size, localization, and basicity, they also seem to display a similar system of compensatory regulation which complicates genetic deletion studies. When the gene for TP1 was deleted in mice, it was shown to produce a number of infertile males with subtle abnormalities in sperm morphology which often led to reduced sperm motility (Yu *et al.*, 2000). More drastic effects may have been prevented, however, by the up-regulation of TP2, which was found to constitute 91% of the basic protein compliment of the nucleus in step 13 spermatids, compared to <4% histone (Yu *et al.*, 2000). Deletion of the TP2 gene gave even less drastic phenotypes: mutant mice were fertile but did tend to produce smaller litters, while microscopy revealed some incomplete chromatin condensation as well as tail abnormalities (Zhao *et al.*, 2001; Table 1). Levels of TP1 in TP2-null mice were seen to increase to 87% of the basic nuclear proteins (Zhao *et al.*, 2001). The subsequent creation of double-null mutants in these transition proteins led to observable defects in chromatin condensation, as well as sterility in affected mice (Zhao *et al.*, 2004). However, as in the case of the H1T2 deletion, the sperm still produced efficient fertilization via intracytoplasmic injection (Zhao *et al.*, 2004).

During the final few steps of the differentiation phase of spermatogenesis, the transition proteins are replaced by a final set of basic proteins called protamines to prepare the DNA for delivery to an oocyte. Protamine 1 (P1 or PRM1) and protamine 2 (P2 or PRM2) are almost exactly the same molecular weight as TP1 and TP2, respectively, but are composed of >50% arginine, with little or no lysine present (Lewis *et al.*, 2004; Table 1; Fig. 1). While none of these four proteins are classified as histones, it has been demonstrated that the emergence of protamines in chordates was likely the result of a frameshift mutation in the tail of a *bone fide* histone H1 which converted the lysine-rich protein to an arginine-rich one (Lewis *et al.*, 2004). While no such phylogenetic link has been established for the transition proteins, it appears that they are a fairly recent addition to the basic nucleoprotein complement of sperm, as fish and birds lack this class of proteins completely and can be seen to undergo a transition directly from histone-containing chromatin to protamine-associated DNA (Yu *et al.*, 2000). In contrast, annelids and echinoderms maintain the nucleosomal conformation of chromatin in sperm and lack both transition proteins as well as protamines (Raja and Renkawitz-Pohl, 2005). Following step 14 in mice, protamines can be seen to have almost completely replaced the transition proteins, making up 74% of the basic protein extractable from spermatids, compared to only 22% transition proteins and <4% histones (Yu *et al.*, 2000). During this time, the DNA forms a fiber around which the protamines wrap, which comes to resemble a stack of doughnuts (Raja and Renkawitz-Pohl, 2005). This ultra-condensed conformation of the DNA is important for both sperm hydrodynamics as well as protecting the genome from physical and chemical damage as it journeys out into the world. From step 15 on, HILS1, as well as the two transition proteins, are no longer detectable in the nucleus (Yan *et al.*, 2003, Fig. 1). In contrast to linker histones as well as the transition proteins, disruption of a single allele of either protamine discussed here demonstrated haploinsfficiency and led to the production of sterile males (Cho *et al.*, 2001). Morphological abnormalities such as sperm with their flagella tightly wrapped around their heads or having elongated heads with reduced ventral flexure were common in mutants, as were indicators of incomplete chromatin condensation (Cho *et al.*, 2001). Finally, after 1.5 to 2 months of development, the spermatids complete step 16 in the mouse and are ready to be released as mature spermatogonia.

Broadening our base

Since this review has focused on mammalian development, namely that of the rat and mouse, up until this point, we will attempt to continue to describe the following events with these organisms in mind. However, discussion of linker histone transitions during both oogenesis and embryogenesis will necessitate the inclusion of data from a much wider array of organisms. While we have pointed out earlier that it has been over 30 years since the first testis-specific linker histone was reported in both rats and

mice, it took until the early 21st century before the first oocyte-specific linker histone was described in mice, the first in any mammalian system (Tanaka *et al*., 2001). Ironically, the first description of an oocyte-specific linker histone actually predated the original discovery of a testis-specific H1 subtype, when a «cleavage stage» histone was characterized in sea urchin (Ruderman and Gross, 1974). This was soon followed by descriptions of histone subtype switches during embryo development in such diverse organisms as the mud snail, *Ilyanassa obsoleta*; two frogs, *Xenopus laevis* and *Bufo japonicus*; the midge, *Chironomus thummi*; mouse; the zebrafish, *Danio rerio*; and, finally, in humans as well (Flenniken & Newrock, 1987; Smith *et al*., 1988; Ohsumi and Katagiri, 1991; Trieschmann *et al*., 1997; Tanaka *et al*., 2001; Wibrand and Olsen, 2002; Muller *et al*., 2002; Tanaka *et al*., 2003, Fig. 4). Confusingly, most of these oocyte-specific linker histones were called by a different name in the literature, including CS-H1, B4, H1X, H1 I-1, H1M, and H1oo (which has recently been altered to H1foo to denote its status as a member of the histone H1 «family», instead of a *bone fide* histone H1, a renaming scheme that has also been applied to its close relative, H1°). Since studies of oocyte-specific histones have primarily focused on *Xenopus laevis*, in which we find histone B4, this will be the most common organism which we now introduce into our discussion.

Oogenesis

While the process of spermatogenesis is quite similar throughout all vertebrates, the details of oogenesis differ considerably, depending on the reproductive strategies of the organism in question (Carlson, 1996). The development of oocytes does share certain similarities with spermatogenesis, however, consisting of similar stages up until the differentiation phase, which is not found in oogenesis, *per se*. This is not to say that oocytes do not undergo differentiation but that they do so concurrently with their meiotic phase, although oocyte development does lack the scope of morphological changes which must take place in sperm. The oocyte is easily the largest and most complex cell that is produced by an animal, and has the distinction of being the only cell for which differentiation is not a terminal condition (Clarke *et al*., 1998). Perhaps because of this lack of a separate differentiation phase, the linker histone transitions which take place during oogenesis are not as complex as those observed for spermatogenesis, involving only the replacement of somatic linker histones with oocyte-specific ones. As we have seen, two out of three of the testis-specific linker histones appear in the differentiation phase of spermatogenesis, in addition to the two classes of non-histone basic nucleoprotein, transition proteins and protamines. In oocytes, the transition to a tissue-specific linker histone, like in the case of histone H1t, appears to correlate with the advent of the meiotic phase and may involve the formation of a looser chromatin structure during this time. Since mitosis is a key feature in the cell cycle of somatic cells, it should come as no surprise that the mitotic phase of both spermatogenesis and oogenesis is characterized by the presence of somatic linker histone types.

Mitotic phase

In females, PGCs destined to form oocytes migrate to the developing ovaries, where they undergo mitosis during months 2-7 of gestation in humans to produce about 6 million primary oocytes. The oocytes are reported to have a complement of somatic linker histones at this stage, but these gradually disap-

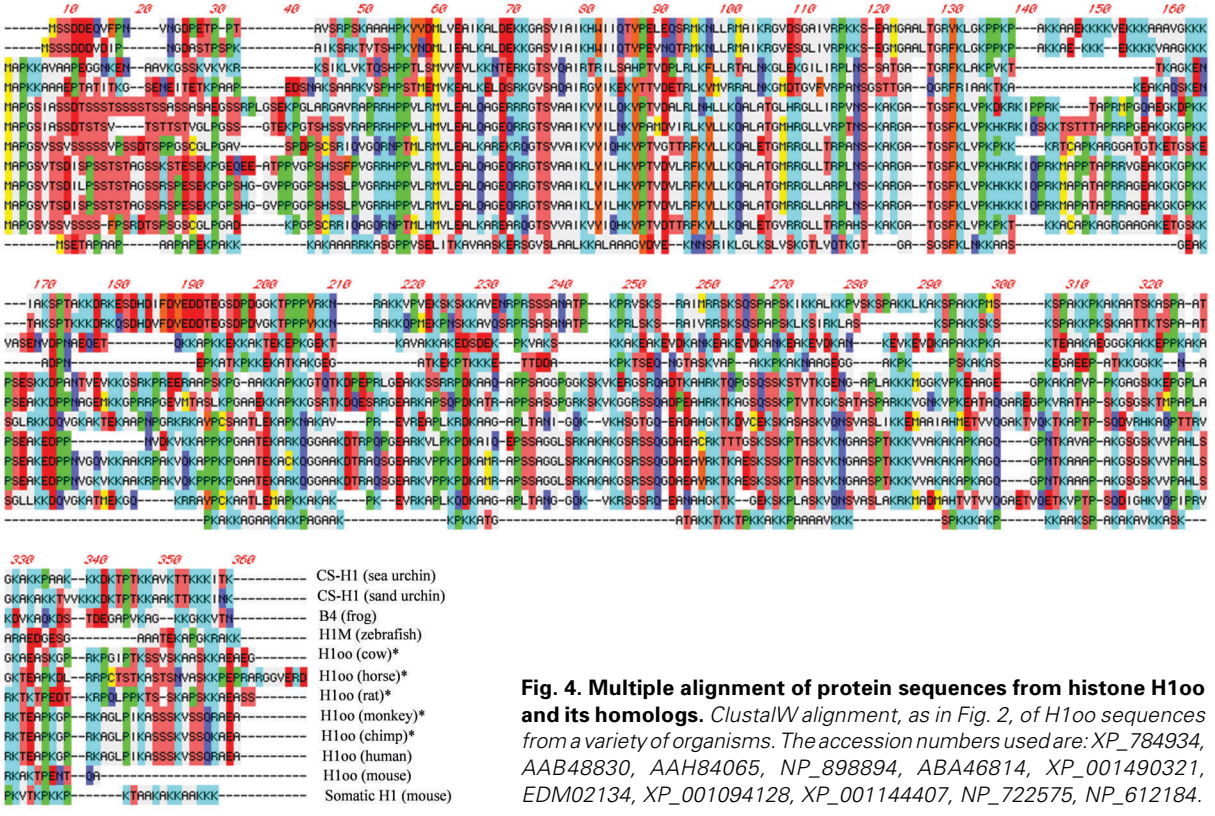

Fig. 4. Multiple alignment of protein sequences from histone H1oo and its homologs. *ClustalW alignment, as in Fig. 2, of H1oo sequences from a variety of organisms. The accession numbers used are: XP_784934, AAB48830, AAH84065, NP_898894, ABA46814, XP_001490321, EDM02134, XP_001094128, XP_001144407, NP_722575, NP_612184.*

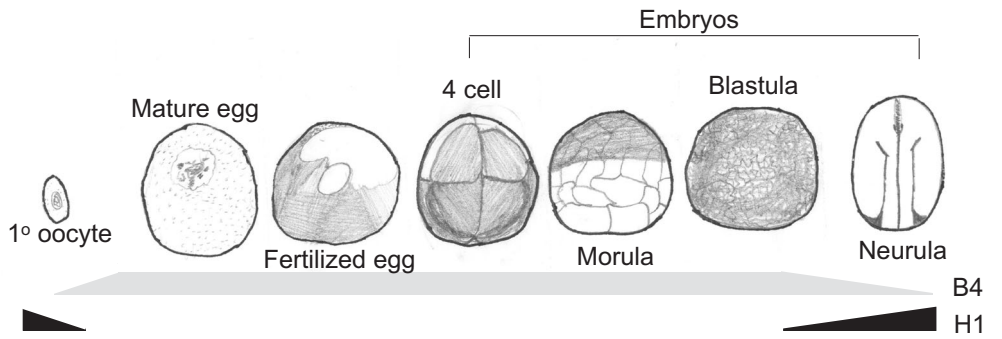

Fig. 5. Morphological changes during oogenesis and embryogenesis, along with known linker histone transitions. *An overview of the major developmental changes which occur during the formation of oocytes in* Xenopus*, as well as during the early stages of embryogenesis. For simplicity, not all stages are shown. Morula, blastula, and neurula correspond to stages 6.5, 9, and 19 of embryogenesis, respectively. Bars representing histone levels are as in Fig. 1, B4 levels are indicated by a gray bar, while somatic H1 levels are indicated by a black bar. Illustration by Justin Godde.*

pear after they exit the mitotic phase (Clarke *et al.*, 1997). In contrast, the primordial cells have been reported to contain low levels of oocyte-specific linker histone expression, while this protein was found to be abundant in primary oocytes (Tanaka, M., *et al.*, 2005, Fig. 5). While the *Xenopus* B4 protein if often described in the literature as appearing early in the meiotic phase, there no hard evidence of its absence in late-mitotic primary oocytes, early studies having only checked for B4 in this stage or later (Smith *et al.*, 1988). Regardless of exactly when this protein first appears, suffice it to say that the oocyte-specific linker histone appears to be important for the transition from the mitotic to meiotic phase in both mammals and amphibians.

Meiotic phase

While spermatocytes undergo an exceptionally long prophase in their first meiotic division, primary oocytes become arrested at this phase in most animals, remaining in this state for vastly different amounts of time. In *Xenopus*, meiotic arrest lasts a little over a year, while in humans it lasts at least until puberty but can extend for nearly 50 years (Carlson, 1996). Unlike spermatocytes, however, oocytes progress through the leptotene, zygotene, and pachytene stages fairly quickly, and become arrested in the diplotene stage, which is normally quite brief in spermatocytes. The oocyte is surrounded at this time by a layer of protective follicular cells, and can be referred to as a primordial follicle (Carlson, 1996).

This is not to say that the oocyte lies completely dormant during diplotene arrest. During this phase, the oocyte begins the process of maturation, somewhat analogous to the differentiation phase of spermatogenesis. Here, chromosomes can be seen to decondense and become transcriptionally active following homologous recombination (Carlson, 1996). In amphibians, birds, and reptiles, the chromosomes take up an unusual conformation, called lampbrush chromosomes, that contain large chromatin loops which extend outward from the chromosomes. The lampbrush configuration, or simply chromosome decondensation in other animals, is coupled with an increase in transcription at all levels, producing large amounts of mRNA, rRNA, and tRNA (Carlson, 1996). A typical model for this transcription in *Xenopus* is the study of 5S rRNA expression, the number of genes of which have been increased by DNA amplification by this point (Crane-Robinson, 1999). The nucleus of the primary oocyte swells as it fills with RNA, especially in organisms which contain lampbrush chromosomes. In *Xenopus*, this large nucleus is called the germinal vesicle, and this stage of oogenesis is also referred to as GV (Carlson, 1996). Coupled with the enlargement of the nucleus, the cytoplasm of certain animals such as *Xenopus* begins to enlarge at this point, forming a yolk that is filled with lipids, polysaccharides, and RNA that has been transported there, some of which undergoes translation, but much of which is stored in an inactive form. Mammals, however, do not develop an appreciable yolk; mature human oocytes, for instance, reach a size of only 0.1 mm across, only one quarter the diameter of the nucleus in a *Xenopus* oocyte (Carlson, 1996; Fig. 5). Since it is during the diplotene stage of meiosis that oocyte-specific histones have their first discernable function in oogenesis, we will take up a discussion of them now.

In contrast to histone HILS1, the shortest of the linker histones to be characterized, the oocyte-specific histones are the longest H1 histones to be described, mostly owing to their exceptionally long C-terminal tails (Tanaka, Y., *et al.*, 2003, Fig. 4). Like H1t, the lysine to arginine ratio is reduced below that of the somatic histones in the oocyte-specific histone, being approximately 2:1 in both mammalian forms of H1oo described (Tanaka *et al.*, 2001; Tanaka, Y., *et al.*, 2003; Table 1). Phylogenetic analysis of this protein, however, shows that it is more closely related to H1T2 and H1º than it is to H1t (Yan *et al.*, 2003). Another feature that H1oo shares with H1T2, but not H1t, is the presence of potential sites of phosphorylation (Tanaka *et al.*, 2001). One unique feature of oocyte-specific linker histones is a relatively high level of acidic amino acids, compared with somatic histones. This is especially evident in histone B4, which has 36 aspartic and glutamic acid residues, almost all of which are localized in the extended C-terminal tail (Fig. 4). This is nearly half the number of basic amino acids contained throughout the protein. Because of these primary sequence differences with somatic H1, as well as the suspicion that B4 played role a significantly different role *in vivo* than somatic histones, our laboratory has devised a number of *in vitro* studies to study the differential effects of B4 binding to chromatin.

Using a dinucleosome which had been reconstituted *in vitro* on two 5S rRNA genes, the binding of B4 was compared to that of somatic H1 (Ura *et al.*, 1996). Although both linker histones were able to protect internucleosomal linker DNA from digestion by nucleases, the stability of these structures varied (Ura *et al.*, 1996). We developed a new chromatin assembly system using a linker histone chaperone, and subsequent studies revealed differences in these two linker histones' ability to restrict the accessibility of nucleases to the protected linker DNA, as well as revealing that histone B4 allows chromatin to be remodeled by ATP-

dependent chromatin remodeling factors, while H1 does not (Saeki *et al.*, 2005). We have continued these studies using *in vitro* reconstituted 12-mer nucleosomal arrays on a synthetic nucleosome positioning sequence which allows for differences in chromatin folding to be discerned. Using analytical centrifugation to measure levels of chromatin compaction, we have shown that histone H1 has a much wider array of interactions with chromatin, being able to either relax or condense chromatin arrays in a concentration dependent fashion (Godde and Ura, unpublished results). B4, on the other hand, was seen to display a much narrower range of interactions, usually folding chromatin arrays to a lesser extent, compared to the same concentration of H1.

Other groups have performed *in vivo* studies to address this same question. Recently, the mouse mammary tumor virus (MMTV) promoter was reconstituted in *Xenopus* oocytes to address the differential effect of linker histone binding to chromatin templates (Belikov *et al.*, 2007). While the expression of H1A, the major somatic subtype in *Xenopus*, could be demonstrated as having been incorporated into chromatin, the effects that it had on hormone-dependent MMTV transcription proved concentration dependent. Subsaturating levels of H1 expression were seen to enhance transcription of this construct, while this hormone-dependent stimulation was lost at higher levels of expression (Belikov *et al.*, 2007). Curiously, expression of histone B4, in contrast, did not affect either chromatin architecture or MMTV expression, suggesting a somatic histone H1 specific function in gene activation. Furthermore, the B4 protein failed to compete with histone H1 binding to chromatin *in vivo* (Belikov *et al.*, 2007).

This failure to compete with somatic H1 leads us to ponder whether such a direct competition ever takes place *in vivo* during diplotene arrest, the time that is presumably crucial for such a replacement to take place. We have described the gradual disappearance of somatic H1 following the mitotic stage of oogenesis, and the available evidence supports its nearly complete absence from the oocyte by the time of meiosis arrest. While mRNA encoding for all six of the somatic subtypes of H1 could be detected in GV-stage oocytes of *Xenopus*, none of their corresponding proteins were found to be present using immunological techniques, with the exception of histone H1° (Hock *et al.*, 1993; Clarke *et al.*, 1997; Clarke *et al.*, 1998; Fu *et al.*, 2003). It was later shown that injected somatic H1 was not able to localize properly in mouse oocyte chromatin, compared to H1oo (Becker *et al.*, 2005). Since the B4 protein has been shown to associate with the histone chaperone NAP-1 in *Xenopus* oocytes, the deposition of particular linker histone variants *in vivo* is likely to be regulated by histone H1-chaperone complexes rather than involve the direct replacement of subtypes on a large scale (Shintomi *et al.*, 2005; Fig. 5).

Once cells, now called secondary follicles, are released from diplotene arrest, whether by the advent of puberty or by the completion of the first stages of maturation, they complete prophase I fairly rapidly and move on to the metaphase, where the chromosomes align down the middle of the cell. Surprisingly, it is at this stage, not diplotene arrest, that mouse oocytes will arrest in if depleted of H1oo. When GV oocytes were injected with antisense morpholino oligonucleotides against H1oo, two-thirds of the injected cells were seen to arrest in metaphase I (Furuya *et al.*, 2007; Table 1). It is not clear, however, whether the injection of another H1 subtype could rescue this phenotype, as injection of exogenous H1oo was seen to do, future genetic studies will be required to conclude whether H1oo is truly essential for oocyte viability (Furuya *et al.*, 2007). Irregardless, these data suggest that the oocyte-specific histone, while not absolutely required for the completion of prophase I, plays a role in the completion of metaphase. At anaphase I and telophase I which follow, the accompanying cytoplasmic division is unequal, creating the first polar body. It was the absence of such a polar body in oocytes lacking H1oo that first led the above authors to suspect a stage-specific arrest had occurred (Furuya *et al.*, 2007). The oocytes can now be called secondary oocytes, or alternately, tertiary follicles, as they enter their second round of meiosis. As the oocytes progress through meiosis II, they are ovulated in mammals, only to become arrested a second time, this time in metaphase II. This meiotic arrest, however, lasts nowhere near as long as the first one, as cells will be released from it upon fertilization.

Fertilization

After the union of sperm and egg, the protamine-bound sperm chromatin can be seen to decondense as the protamines are replaced by oocyte-specific histones; the swollen engulfed sperm nucleus is now called the male pronucleus (Carlson, 1996; Fig. 5). In sea urchin, where linker histones are not replaced by protamines during spermatogenesis, the sperm H1 can be seen to be phosphorylated at their N-terminal tails before being replaced by the oocyte-specific linker histone (Mandl *et al.*, 1997). The functions that the oocyte-specific linker histones play in the developing embryo at this point have not been determined with any certainty. It has been much easier to establish some of the things that these H1 variants do not do during early embryonic development. Depletion of B4 from *Xenopus* oocyte extracts, for instance, has demonstrated that the presence of this histone is not required for the assembly of morphologically normal pronuclei which are capable of DNA replication (Ohsumi *et al.,* 1993; Dasso *et al.*, 1994). Similar experiments also established that B4 did not contribute to the physiological spacing of nucleosomes (Dimitrov *et al.*, 1994). It has been proposed, however, that the presence of an oocyte-specific histone in embryos may both facilitate the rapid DNA replication which takes place in early embryos as well as be involved in the resetting of the genome to produce a totipotent state (Clarke *et al.*, 1998). We will address the first part of this hypothesis now, but will save the latter part for a bit later in our discussion. In early cleavage stage embryos from *Xenopus*, S-phase has been seen to last only 15 minutes or so, a time which lengthens considerably as B4 is progressively lost during development. The addition of exogenous somatic H1 at this point has been shown to inhibit the replication process by reducing the number of replication forks in sperm nuclei, while leaving the rate of fork progression unchanged (Lu *et al.*, 1998). As in the above-described experiments, however, immunodepletion of B4 had no apparent effect on DNA replication in *Xenopus* egg extracts (Lu *et al.*, 1998). It may be true that an open chromatin conformation is required for prereplication complexes to form on sperm chromatin and that oocyte-specific histone H1 is simply serving a supporting role in chromatin stability.

While the male pronucleus undergoes the replacement of its protamine with oocyte-specific H1, the egg nucleus breaks its

meiotic arrest and extrudes another polar body. Both pronuclei are free to enter S-phase at this point, during which they rapidly replicate their respective sets of DNA (Carlson, 1996). As they begin their first mitoses since early in their respective developmental processes, the pronuclei migrate towards one another in the cell and fuse together before they reach metaphase. The two sets of chromosomes then line up together, fully mixing the maternal and paternal chromosomes prior to separation into two nuclei (Carlson, 1996). Although little or no transcription can be detected during the first embryonic cleavage, histone mRNAs which have been blocked from being translated are apparently activated at this time (Teranishi *et al.*, 2004). Following this point, the model systems we have been using to describe oogenesis differ widely from each other. In mouse, and presumably humans, most of the somatic histones make their reappearance following the next mitotic division, during the four-cell stage of embryogenesis (Fu *et al.*, 2003). In *Xenopus*, however, oocyte-specific histone will remain the predominant linker histone until much later in development, at the mid-blastula transition, or MBT (Dimitrov *et al.*, 1993; Fig. 5). Sea urchin, however, appears to be somewhat intermediate between these two extremes, while a histone called α-H1 replaces CS-H1 fairly quickly after fertilization, it is itself replaced by other somatic variants during the blastula stage (Mandl *et al.*, 1997; Hock *et al.*, 1993).

Somatic nuclear transfer

Whether DNA is introduced into an egg by fertilization or by SNT, the linker histone transitions which take place are quite similar. One proposed role played by oocyte-specific histones is the introduction of a totipotent state (Clarke *et al.*, 1998; Saeki *et al.*, 2005). While this question is difficult to ascertain in naturally fertilized embryos, it is more easily addressed in somatic nuclei which have been introduced into donor eggs in order to clone an organism or to produce embryonic stem cells. It has been fifty years since *Xenopus* became the first animal to be cloned using this technique, a finding that was largely ignored by the general public until Dolly the sheep ushered in the advent of mammalian SNT (Gurdon *et al.*, 1958; Gurdon, 2006). One main difference between SNT and fertilization is that somatic H1, not protamines, from the transferred nucleus must be replaced directly by oocyte-specific histones. Experiments using SNT have led to a greater understanding of how this replacement process takes place. First, linker histone replacement can be seen to occur quite quickly-more than 80% of transferred mice nuclei displayed intense positive staining for H1oo only 5 minutes after injection (Gao *et al.*, 2004). Since the nuclear envelopes of transferred nuclei do not break down for approximately one hour, it does not appear that this is a prerequisite for histone replacement (Gao *et al.*, 2004). Furthermore, the linker histone transitions observed appeared to be donor nucleus-independent, even to the extent that a mammalian nucleus introduced into *Xenopus* egg extracts underwent replacement with histone B4 (Miyamoto *et al.*, 2007). It has been demonstrated that a molecular chaperone called nucleoplasmin acts in the selective removal of linker histones from donor nuclei (Dimitrov and Wolffe, 1996). Nucleoplasmin was later shown to play a role in nuclear reprogramming in mice as well (Burns *et al.*, 2003).

Another finding in *Xenopus* was that histone H1^{o} was released from donor chromatin preferentially in comparison with other somatic linker histones (Dimitrov and Wolffe, 1996). It is unclear why this particular linker histone shows preferential release, especially since it has been known for its relatively high affinity for DNA binding (Wellman *et al.*, 1999). This finding, does, however, resolve seemingly contradictory results recently obtained from two different groups using a very similar approach. Both groups studied histone replacement using FRAP, fluorescence recovery after photobleaching, in mice somatic nuclei which had been introduced into oocytes by SNT. One group came to the surprising conclusion, based on what we have discussed above, that histone H1oo binds to chromatin more tightly than somatic linker histones, while the earlier group had reached precisely the opposite conclusion (Teranishi *et al.*, 2004; Becker *et al.*, 2005). This inconsistency could possibly be explained by the specific somatic H1 that was used in comparison with H1oo. The early experiments compared the mobility of H1oo with that of H1.2, reporting a $t_{1/2}$ of recovery of about 22 seconds for the former and 29 seconds for the latter (Teranishi *et al.*, 2004). Hence, the conclusion that H1oo bound less tightly to chromatin than somatic H1 was upheld. The later studies, however, compared H1^{o} mobility with H1oo, reporting a $t_{1/2}$ of about 6 seconds for the former and 32 seconds for the latter (Becker *et al.*, 2005). Since the mobility of H1oo in the two studies is roughly comparable, one might conclude that the H1^{o} in these studies is actually binding more loosely to chromatin than expected, rather than the H1oo binding more tightly. Although the study which used H1^{o} for comparison was able to repeat their findings using somatic cells in addition to oocytes, a recent study which used FRAP to measure the mobility of H1^{o} in somatic cells confirmed a relatively fast rate of exchange for this histone (Meshorer *et al.*, 2006).

Later development

As development continues after fertilization or SMT, a series of synchronous cell divisions lead to an embryo which looks like a small mulberry, the morula stage (Carlson, 1996; Fig. 5 & 6). In *Xenopus*, embryos between the 16- and 64-cell stages are considered to be morula. After this, the cell divisions begin to lose their synchronous character and the embryo soon develops a central cavity called a blastocoel (Carlson, 1996). The embryo has now entered the blastula stage, a process which occurs about 4 hours post fertilization in *Xenopus*. As the blastula grows towards the next major stage, the gastrula, in which the process of gastrulation begins, three major regions can be discerned: 1) cells forming the roof of the blastocoel which will go on to form the ectoderm, 2) cells in the interior of the blastocoel which will go on to form the endoderm, and 3) cells in an equatorial ring which will go on to form the mesoderm (Carlson, 1996). These primary germ layers will then undergo further restriction once the process of neural induction begins, bringing with it the formation of the neurula (Fig. 5 & 6).

It is about 6 hours into development, and roughly halfway to the gastrula stage, that the *Xenopus* embryo reaches its final major linker histone replacement, the mid-blastula transition. This histone replacement, like the others we have discussed, takes place gradually. Ever since the early stages of embryonic cleavage, the level of B4 histone can be seen to slowly decrease, while the level of somatic histones slowly increase, in what has been observed

at being a roughly reciprocal relationship (Ohsumi and Katagiri, 1991; Dimitrov *et al.*, 1993; Saeki *et al.*, 2005; Fig. 6). Certain embryonic genes, such as the oocyte-type 5S rRNA gene, are down-regulated at this time, while others, such as the somatic 5S rRNA gene, are up-regulated. *In vitro* studies demonstrated that the addition of somatic H1 to chromatin resulted in chromatosomes being positioned directly over oocyte-type genes, thereby occluding transcription factor binding, while the same histone led to a different chromatosomal position on the somatic-type genes, leaving them exposed to factor binding (Ura *et al.*, 1995; Sera *et al.*, 1998). As expected, the injection of ribozymes which disrupt the histone H1A gene has been shown to prolong the expression of the oocyte 5S rRNA gene during MBT, while the concurrent overexpression of another somatic histone had the opposite effect (Steinbach *et al.*, 1997). A link between oocyte-specific histones and pluripotency was supported by the observation that the accumulation of somatic H1 was rate limiting for the loss of mesodermal competence in cells from the blastocoel cap which would normally have become ectoderm, but could be induced to form mesoderm by supplementing them with a specific growth factor (Steinbach *et al.*, 1997). Again, it may be the overall chromatin conformation at this point that is most important and not the actual histone H1 variants themselves. Assuming that the binding of oocyte-specific linker histone leads to a more open chromatin state, the same effect could presumably be produced by the hyperdynamic binding of somatic H1. Indeed, it has recently been demonstrated that histone H1° binds loosely to chromatin in mouse ES (embryonic stem) cells and that cells in which the dynamic exchange of H1° is restricted display differentiation arrest (Meshorer *et al.*, 2006). These studies compared the mobility of H1° in ES cells to that of H3 and H2B, using FRAP, and found that the $t_{1/2}$ of recovery was about 8 seconds for the linker histone, compared to 50 seconds for the core histones (Meshorer *et al.*, 2006). These levels of mobility were then seen to decrease in differentiated cells (Meshorer *et al.*, 2006).

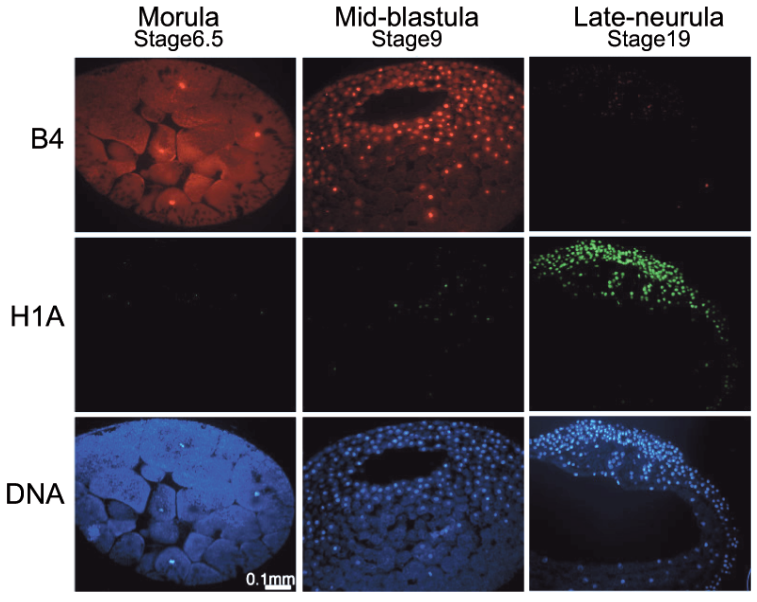

Fig. 6. Fluorescence microscopy demonstrating linker histone transitions during *Xenopus* embryogenesis. *Cytological sections of* Xenopus *embryos at the indicated stage of development. Staining is with antibodies to either B4 or H1A, or with DAPI, which stains the DNA. Figure derived from Saeki* et al.*, copyright (2005) National Academy of Sciences, USA., used with permission.*

Summary

We have portrayed the process of development as a series of linker histone replacements. Exactly how, or why, these replacements take place is still unclear, but the fact that they do occur throughout a wide range of metazoans suggests that they likely serve an important role. While we have primarily focused on the effects of linker histone variants on chromatin structure during this review, we realize that H1 may play functional, as well as structural, roles in development which have yet to be elucidated. One unexpected functional role for H1 that has been recently described is its ability to convey signals in the cell. In this case, the presence of H1.2 in the cytoplasm acted to induce apoptosis by promoting the release of cytochrome C from the mitochondria (Konishi *et al.*, 2003). Previously unforeseen structural roles for linker histones have recently been described as well. For instance, H1 has been shown to exhibit a structural role in microtubule nucleation and organization in acentriolar plants (Hotta *et al.*, 2007; Nakayama *et al.*, 2008). It may thus turn out that linker histones play a number of roles in the cell which are independent of their incorporation into chromatin. We have tried to speculate above what the linker histone replacements in question may mean to chromatin structure, but recognize that more work will be required to elucidate their precise role in development.

We have detailed no less than seven replacement events, involving both linker histone variants as well as certain basic non-histone proteins, which occur in the context of the male genome as it progresses through spermatogenesis, participates in the fertilization of an egg, and finally develops into a sexually mature organism that is able to begin the whole developmental process over again. Female genomes, in contrast, appear to go through a much simpler change in linker histone variants as they oscillate between somatic and oocyte-specific H1s. Certain similarities between the sexes are of note, however. For instance, both types of germ cells start development as PGCs with a complement of somatic histone H1. These then take separate paths in males and females to become mature sperm and egg cells, both becoming arrested during their respective meiotic phases and undergoing a transition from somatic linker histones to gamete-specific ones. In both cases, the gamete-specific linker histones have been noted for their lower affinity for nucleosome binding as well as their reduced ability to condense chromatin. The differentiation/maturation phases of these two gametes then take widely different amounts of time, with sperm becoming mature in months but eggs often taking decades. Upon fertilization, the sperm chromatin rapidly goes through its penultimate linker histone transition, this time accepting the H1 complement of the egg, thereby allowing the newly combined genome to ready itself for the process of embryogenesis. It is this replacement event which appears to be critical for «resetting» the genome during the establishment of a totipotent state and has therefore been a primary focus of research in our laboratory. The replacement of somatic H1 with oocyte-specific H1 can

also be seen to accompany SNT, which further attests to the importance of this transition. A final replacement back to somatic subtypes then occurs with varying rapidity, depending on the species, but is often concomitant with the cellular differentiation that is associated with later stages of development.

Acknowledgements

We wish to thank Dr. Y. Kaneda, as well as members of our laboratory, Drs. H. Saeki and K. Ohsumi for helpful discussions. This work was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan and the Takeda Science Foundation.

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