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Orchestrating the Specific Assembly of Centromeric Nucleosomes

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Abstract

Centromeres are chromosomal loci that are defined epigenetically in most eukaryotes by incorporation of a centromere-specific nucleosome in which the canonical histone H3 variant is replaced by Centromere Protein A (CENP-A). Therefore, the assembly and propagation of centromeric nucleosomes are critical for maintaining centromere identity and ensuring genomic stability. Centromeres direct chromosome segregation (during mitosis and meiosis) by recruiting the constitutive centromere-associated network of proteins throughout the cell cycle that in turn recruits the kinetochore during mitosis. Assembly of centromere-specific nucleosomes in humans requires the dedicated CENP-A chaperone HJURP, and the Mis18 complex to couple the deposition of new CENP-A to the site of the pre-existing centromere, which is essential for maintaining centromere identity. Human CENP-A deposition occurs specifically in early G1, into pre-existing chromatin, and several additional chromatin-associated complexes regulate CENP-A nucleosome deposition and stability. Here we review the current knowledge on how new CENP-A nucleosomes are assembled selectively at the existing centromere in different species and how this process is controlled to ensure stable epigenetic inheritance of the centromere.

In all eukaryotes centromeres serve as a site of kinetochore formation that facilitates faithful chromosome segregation during cell division. Centromeres in most species are characterized by the presence of unique nucleosomes containing the histone H3 variant Centromere Protein A (CENP-A). However, different organisms employ distinct strategies to specify centromere location. Budding yeast contain point centromeres, the location of which is determined by the presence of approximately 150 bp domain with three distinct DNA sequences: CDEI, CDEII, and CDEIII (Clarke and Carbon 1980; Fitzgerald-Hayes et al. 1982). In budding yeast, these sequences are sufficient for the establishment of a functional centromere. However, the wide variation of centromere DNA repeat sequences across species, and indeed the lack of DNA repetitive elements in several species suggest that DNA

sequence elements may not be critical for centromere function in higher eukaryotes (Allshire and Karpen 2008; Shang et al. 2010; Wade et al. 2009). Moreover, the existences of neocentromeres and pseudodicentromeric chromosomes (Scott and Sullivan 2013) strongly suggest that centromeres do not depend on the underlying DNA sequence for their inheritance but are epigenetic loci that are stably inherited through epigenetic processes. Indeed, in higher eukaryotes the centromeric chromatin is defined by epigenetic chromatin features, primarily by the presence of a centromere-specific CENP-A histone variant, rather than underlying DNA sequence. CENP-A specification of epigenetic centromeres means that the process of nucleosome assembly is a key event in inheritance of the locus.

All histone H3 variants employ distinct mechanisms, facilitated by histone chaperones, which selectively recognize them upon synthesis and escort to the site of nucleosome assembly (Filipescu et al. 2014). Similarly, CENP-A uses its own specific machinery that orchestrates the spatiotemporal assembly of centromeric chromatin during the cell cycle. In humans new CENP-A incorporation is a multistep mechanism that involves identification of centromeric chromatin for new CENP-A incorporation, deposition of newly synthesized CENP-A/H4 and stabilization of CENP-A nucleosomes. Each of those steps requires the activity of multiple protein factors which work together to ensure that CENP-A nucleosomes are deposited specifically at the centromeric domain, at the correct time and only once per cell cycle. Mechanisms regulating CENP-A incorporation are well conserved across eukaryotes and here we summarize the current knowledge on the processes regulating new CENP-A deposition in different species.

1 Histone Chaperones and Centromere Assembly

Incorporation of histones into the chromatin requires assembly factors or chaperones that work together to facilitate nucleosome deposition (Burgess and Zhang 2013; Ransom et al. 2010). Histone H3 variants use their specific independent chaperone complexes that govern a selective recognition and facilitate their deposition in replication-dependent (H3.1 variant) or replication-independent (H3.3 and CENP-A variants) nucleosome assembly pathways (Sarma and Reinberg 2005; Szenker et al. 2011; Weber and Henikoff 2014). The major histone variant H3.1 is deposited into newly replicated naked DNA during DNA replication via the CAF-1 complex that include p150, p60, and p46/48 (Tagami et al. 2004; Tyler et al. 1999, 2001). The H3.3 variant is regulated by two chaperone complexes distinct from the H3.1 replication-dependent chaperones responsible for H3.1 deposition. The HIRA chaperone is devoted to the genome-wide deposition of histone H3.3 at active and repressed genes (Chow et al. 2005; Goldberg et al. 2010; Lewis et al. 2010; Mito et al. 2005; Szenker et al. 2011; Tagami et al. 2004; Tamura et al. 2009). DAXX also acts as a chaperone for H3.3 and mediates H3.3 deposition at telomeric and pericentric heterochromatin in conjunction with the H3K9-binding protein ATRX (Goldberg et al. 2010; Lewis et al. 2010).

Similar to the other H3 variants, the centromere-specific histone H3 variant CENP-A interacts with a dedicated chaperone prior to deposition into chromatin. Prenucleosomal human CENP-A associates with the Holliday junction recognition protein (HJURP) (Dunleavy et al. 2009; Foltz et al. 2009; Shuaib et al. 2010). HJURP is necessary for incorporation of vertebrate CENP-A into the centromeric chromatin and is recruited to

centromeres in early G1, when new CENP-A assembly is occurring (Fig. 1) (Bernad et al. 2011; Dunleavy et al. 2009; Foltz et al. 2009; Jansen et al. 2007). Suppression of HJURP completely abolishes new CENP-A deposition, results in errors in kinetochore assembly and ultimately leads to a high rate of chromosome segregation defects (Dunleavy et al. 2009; Foltz et al. 2009).

The centromere targeting domain (CATD) of CENP-A is sufficient to determine the centromeric deposition of CENP-A. The CATD domain spans loop 1 and the $\alpha 2$ helix of CENP-A and when replaced with corresponding domain within canonical H3.1 was demonstrated to confer both HJURP binding and centromeric localization (Black et al. 2007; Foltz et al. 2009). His 104 and Leu112 residues within the CATD C-terminal region, together with either Asn85 or Gln89 within CATD N-terminus, are sufficient to confer HJURP binding, but not sufficient to facilitate centromere incorporation (Bassett et al. 2012).

HJURP specifically recognizes the CATD domain of CENP-A through its N-terminal CENP-A binding domain (Fig. 2). The CENP-A binding domain of HJURP shares homology with the yeast Scm3 proteins that also act as CENP-A (Cse4, Cnp1)-specific chaperone (Figs. 2 and 3) (Camahort et al. 2007; Mizuguchi et al. 2007; Pidoux et al. 2009; Sanchez-Pulido et al. 2009; Stoler et al. 2007; Williams et al. 2009). Although the mechanism of centromere inheritance between budding yeast and humans is very different, both systems are dependent on a CENP-A-specific histone chaperone. HJURP binds CENP-A through the conserved Scm3 domain. A number of residues within yeast Scm3 were proposed to be essential for CENP-A^{Cnp1} incorporation including Leucine 56 and Leucine 73. The fact that those key residues required for CENP-A^{Cnp1} deposition are conserved as hydrophobic amino acids in other eukaryotes, including humans, implies the mechanism by which CENP-A is selectively recognized and deposited at centromeric chromatin by its chaperone is common between yeast and humans (Cho and Harrison 2011; Pidoux et al. 2009).

In contrast to HJURP, which is recruited to centromeres with a refined temporal window when new CENP-A nucleosomes assembly occurs, the fission yeast Scm3 protein remains associated with the centromere through most of the cell cycle (Pidoux et al. 2009). This localization may provide a mechanism to insure the reassembly of CENP-A^{Cnp1} in the event of centromeric chromatin disruption, or to block the ubiquitination and degradation of centromeric CENP-A^{Cnp1}. Alternatively Scm3 may provide additional function at the centromere beyond CENP-A^{Cnp1} deposition.

The crystal structures of both yeast Scm3/CENP-A^{Cse4}/H4 and human HJURP-Scm3/CENP-A/H4 complexes demonstrate that the association of CENP-A (Cse4) with its chaperone prevents CENP-A/H4 tetramer formation, and precludes spontaneous DNA interactions by the histone complex in the prenucleosomal form (Cho and Harrison 2011; Hu et al. 2011). Vertebrate HJURP is much larger than its yeast orthologue Scm3, and contains several domains that are absent from both the *S. pombe* and *S. cerevisiae* orthologues (Fig. 3) (Sanchez-Pulido et al. 2009). Similar to *S. cerevisiae* Scm3, human HJURP was demonstrated to mediate an interaction with DNA through its “mid” domain (HMD), which

is required for new CENP-A deposition (Fig. 2) (Muller et al. 2014; Xiao et al. 2011). It is not known whether in addition to its ability to bind DNA, HJURP also has a capacity to interact with RNA. Given the evidence that RNA plays a role in centromere specification and HJURP recruitment it is an outstanding question that awaits future studies (Bergmann et al. 2011; Quenet and Dalal 2014a).

Centromeric recruitment of HJURP is independent of CENP-A binding and is mediated by the HJURP carboxyl terminal domain 1 (HCTD1) (Fig. 2) (Wang et al. 2014; Zasadzinska et al. 2013). The HJURP carboxyl terminal domain 2 (HCTD2) serves as a homo-dimerization interface and facilitates HJURP self-association, consistent with formation of the budding yeast Scm3/CENP-A^{Cse4}/H4 hexamer and Scm3 self-association in fission yeast. In those species the multimerization mediated by the CENP-A chaperone is required for new CENP-A deposition (Mizuguchi et al. 2004; Pidoux et al. 2009; Wang et al. 2014; Zasadzinska et al. 2013). This evidence provides a mechanism by which prenucleosomal HJURP complex brings two CENP-A molecules to the site of CENP-A deposition consistent with the CENP-A nucleosomes forming an octamer. Alternatively, one HJURP present in the prenucleosomal complex brings newly synthesized CENP-A/H4 heterodimer, and the other HJURP molecule can recognize CENP-A present within centromeric chromatin, consistent with the hemisome hypothesis (Wang et al. 2014; Zasadzinska et al. 2013).

The proposed role of the histone chaperone has been to preclude the stochastic interactions between the histone protein and DNA prior to nucleosomes assembly. Consistent with this idea, the interaction of HJURP with the CENP-A/H4 heterotetramer blocks several key residues along the DNA interface of CENP-A (Cse4) (Cho and Harrison 2011; Hu et al. 2011). In addition, histone chaperones are known to facilitate the assembly of histone subunits into nucleosomes. Both Scm3 and HJURP mediate CENP-A (Cse4) nucleosome assembly in vitro (Barnhart et al. 2011; Camahort et al. 2009; Dechassa et al. 2011; Shivaraju et al. 2011). Much consideration has been given to whether the CENP-A nucleosome adopts non-canonical forms which have been reviewed extensively (Black and Cleveland 2011; Quenet and Dalal 2012). Deposition experiments suggest that, while CENP-A may take on varied conformations, the CENP-A chaperone facilitates the formation of octameric nucleosomes with a left-handed wrap of the DNA (Barnhart et al. 2011; Dechassa et al. 2011).

2 Prenucleosomal Posttranslational Modifications and CENP-A Deposition

CENP-A is bound to its chaperone as a heterodimer with histone H4, thus modification of H4 may contribute to CENP-A nucleosome assembly. Indeed, histone H4 is acetylated on K5ac and K12ac within the prenucleosomal complex, and these modifications are necessary for CENP-A deposition (Fig. 1) (Shang et al. 2016).

Human RbAp46 (a.k.a. RBBP7) and RpAp48 (a.k.a. RBBP4) are highly homologous genes whose protein products are present in many chromatin remodeling complexes (Loyola and Almouzni 2004). Mutants of the *S. pombe* homolog of the RbAp proteins, Mis16, cause chromosome segregation defects due to a failure to assemble CENP-A^{Cnp1} nucleosomes (Hayashi et al. 2004). RbAp46/48 co-purified with HJURP in the prenucleosomal CENP-A

complex (Dunleavy et al. 2009; Shuaib et al. 2010). A crystal structure of the Mis16-Scm3-CENP-A^{Cnp1}/H4 complex shows that Mis16 contacts both the Scm3 chaperone and histone H4 (An et al. 2015). Depletion of RbAp proteins reduces HJURP recruitment and new CENP-A deposition (Dunleavy et al. 2009; Shang et al. 2016). K5 and K12 acetylation of the histone H4 bound to CENP-A within the prenucleosomal complex are dependent on RbAp48, and these modifications are required for CENP-A deposition in vivo (Fig. 1) (Shang et al. 2016). In the *Xenopus* system H4K5 and H4K12 acetylation marks in prenucleosomal CENP-A complex are dependent upon HAT1 activity (Shang et al. 2016) which is also required for CENP-A deposition in *Drosophila* (Boltengagen et al. 2016). Therefore, a major role of RbAP48 may be the recruitment of the histone acetyltransferase required for modifying Histone H4. What components may read out the presence of H4 acetylation within the assembly pathway is not known.

RbAp46 and RbAp48 depletion results in reduced HJURP protein levels (Dunleavy 2009) and a second role for these proteins may be in regulating the stability of the CENP-A prenucleosomal complex (Mouysset et al. 2015). RbAP46 forms a complex with the CRL4 ubiquitin ligase, a member of the cullin-RING-ligase family, and DDB1 protein (where DDB1 mediates the association of CUL4 with its substrate-specific receptor—RbAP46) (Lee and Zhou 2007; Mouysset et al. 2015). RbAP46 is required for stabilizing CENP-A protein levels and the CRL4-RbAP46 complex activity promotes efficient new CENP-A deposition in humans (Mouysset et al. 2015). This is in contrast to studies in yeast and *Drosophila*, where the association of CENP-A with the SCF E3-ubiquitin ligase complex leads to CENP-A degradation (see below).

Two different posttranslational modifications of human CENP-A are proposed to be important for CENP-A deposition. These are phosphorylation of serine 68 and ubiquitylation of lysine 124 (Niikura et al. 2015; Yu et al. 2015). Both modifications are located outside of the CATD domain that is sufficient for HJURP binding, and situated on the helix α 1 and helix α 3 of CENP-A, respectively. However, both are proposed to influence HJURP binding to CENP-A. CENP-A lysine 124 (K124) in humans undergoes mono- and diubiquitylation mediated by the CUL4A-RBX1-COPS8 E3 ligase complex (Niikura et al. 2015). Downregulation of any of the CUL4A-RBX1-COPS8 subunits or mutation of Lys124 leads to loss of centromeric CENP-A in mitosis and interphase cells. Mutation of CENP-A lysine 124 weakens the interaction with CENP-A chaperone HJURP.

Phosphorylation at CENP-A-Ser68 is proposed to preclude its interaction with HJURP, negatively regulating new CENP-A deposition. CENP-A Ser68 phosphorylation depends on Cdk1/cyclin B activity during early mitosis and PP1a phosphatase dephosphorylates Ser68 in late mitosis, making CENP-A competent for HJURP binding and new incorporation in the following G1 (Yu 2015). While the phosphomimetic S68Q mutation appears to preclude HJURP binding both in vivo and in vitro (Hu et al. 2011; Yu et al. 2015), Bassett et al. reported that S68Q substitution within CENP-A has no effect on HJURP-mediated targeting and subsequent incorporation into chromatin at non-centromeric sites. Moreover, recombinant CENP-A containing the S68Q mutation forms a complex in vitro with HJURP with similar efficiency when compared to the wild-type form (Bassett et al. 2012). Despite the effects observed in vivo for the S68 and K124 mutations, both mutations are fully able to

rescue CENP-A null cells, suggesting that these modifications are not essential for the process of centromere specification and inheritance (Fachinetti 2016).

In budding yeast, Psh1 prevents ectopic localization of CENP-A^{Cse4} (Hewawasam et al. 2010; Ranjitkar et al. 2010). Psh1 is an E3 ubiquitin ligase that was identified as associated with yeast CENP-A^{Cse4} in immunoprecipitation experiments and characterized as a kinetochore and centromere-associated protein. Psh1 regulates CENP-A^{Cse4} levels by ubiquitinating CENP-A^{Cse4} and targeting it for proteolysis, thus preventing its accumulation outside the centromeric chromatin. Psh1 and Scm3 both recognize the CENP-A^{Cse4}-CATD domain; therefore, Scm3 appears to protect CENP-A^{Cse4} from the Psh1-mediated ubiquitination and subsequent degradation (Hewawasam et al. 2010; Ranjitkar et al. 2010). In flies, CENP-A^{CID} directly interacts with the F-Box Protein Partner of Paired (Ppa), a variable component of a SCF E3-ubiquitin ligase complex in *Drosophila*. Ppa binds CENP-A^{CID} through the CATD domain and regulates its stability (Cardozo and Pagano 2004; Moreno-Moreno et al. 2011; Nakayama and Nakayama 2006; Schuh et al. 2007).

3 Coupling Chaperone Recruitment to Existing Centromeres

Human centromeres range from 0.3 to 5 Mbp in size and account for less than 1% of the chromosome (Cleveland et al. 2003). The restriction of centromeres to a single locus ensures the stable inheritance of centromeres by avoiding situations where multiple centromeres on one chromosome could make attachments to opposing poles and result in chromosome breakage during mitosis.

The recruitment of the CENP-A-specific histone chaperone to the existing centromere is an essential step in epigenetic inheritance. Mis18 is a key adapter protein that mediates the recruitment of the CENP-A chaperone to centromeres in several organisms (Figs. 3 and 4), but is absent from organisms with point centromeres. Mis18 was originally identified in a genetic screen in fission yeast to identify genes required for proper chromosome segregation (Hayashi et al. 2004). spMis18 mutants eliminate CENP-A^{Cnp1} incorporation to centromeres and Mis18 directly interacts with Scm3 to determine its recruitment (Pidoux et al. 2009). In humans, Mis18 exists as a complex comprised of Mis18α, Mis18β, and Mis18BP1 proteins (Figs. 3 and 4). The Mis18 complex is essential for the recruitment of HJURP and CENP-A to the centromeric chromatin due to a direct interaction with the HJURP centromere targeting domain within the HCTD1 (Fig. 2) (Barnhart et al. 2011; Fujita et al. 2007; Nardi et al. 2016; Wang et al. 2014). Mis18 proteins do not require HJURP for recruitment, demonstrating that they are upstream components of the pathway (Barnhart et al. 2011; Bernad et al. 2011). Consistent with studies in yeast, depletion of the Mis18 complex subunits in human cells results in a high rate of chromosome segregation defects and loss of centromeric CENP-A (Fujita et al. 2007). The role of the Mis18 proteins in the CENP-A deposition pathway is evolutionarily conserved, as depletion of Mis18BP1 (KNL-2) homologues in *C. elegans* and *Xenopus* also leads to defects in CENP-A deposition in these species, although, as we discussed below, the pathway has undergone several permutations in different organisms (Maddox et al. 2007; Moree et al. 2011).

Since the Mis18 proteins are required for HJURP recruitment, the key question becomes how the Mis18 protein recognizes the existing centromere. CENP-A nucleosomes recruit the CCAN (constitutive centromere-associated network), a multiprotein complex comprised of 16 subunits, present at the centromere throughout the cell cycle, that serves as a structural core for kinetochore assembly during mitosis (Amano et al. 2009; Cheeseman and Desai 2008; Earnshaw et al. 1986; Foltz et al. 2006; Izuta et al. 2006; McKinley and Cheeseman 2016; Nishihashi et al. 2002; Okada et al. 2006; Saitoh et al. 1992; Sugata et al. 1999). The CENP-C component of the CCAN directly recognizes the CENP-A nucleosome (Carroll et al. 2010; Guse et al. 2011; Kato et al. 2013). New CENP-A nucleosomes within the alpha satellite DNA are assembled directly adjacent to the existing CENP-A (Ross et al. 2016). CENP-C plays a crucial role in recruiting the proteins required for CENP-A deposition (Figs. 1 and 2), and thus links the existing centromere to the assembly of new CENP-A nucleosomes in early G1. CENP-C interacts directly with two proteins within the Mis18 complex, Mis18BP1 and Mis18 β (Dambacher et al. 2012; Moree et al. 2011; Stellfox et al. 2016). CENP-C depletion causes defects in Mis18BP1 and HJURP recruitment and leads to loss of CENP-A chromatin assembly (Dambacher et al. 2012; Moree et al. 2011; Stellfox et al. 2016).

The pivotal role that CENP-C plays in determining the site of centromeric chromatin assembly is exemplified by experiments in chicken DT40 cells, where the endogenous centromere is conditionally removed and the functional kinetochore assembled at an ectopic LacO locus. These experiments show that tethering the LacI-fused HJURP or full-length CENP-C are sufficient to recruit CENP-A in order to establish a functional epigenetic de novo centromere (Hori et al. 2013). Although tethering the CENP-C N-terminus (1–643 aa) in this system is sufficient to recruit microtubule binding proteins and the CPC complex, it fails to incorporate CENP-A nucleosomes (Hori et al. 2013). This is consistent with the identification of the N-terminus of CENP-C as the region of interaction with Mis18BP1 and Mis18 β (Dambacher et al. 2012; Moree et al. 2011; Stellfox et al. 2016). In contrast, CENP-C homologues in yeast (Mif2 and Cnp3) are not essential to facilitate CENP-A deposition (Fig. 3) (Meluh and Koshland 1995, 1997; Westermann et al. 2003).

Additional factors in the CCAN also contribute to directing new CENP-A nucleosome deposition. Depletion of the CENP-HIKM complex in chicken cells compromise the incorporation of newly synthesized CENP-A (Okada et al. 2006). Consistent with this observation, fission yeast CENP-I^{Mis6} and CENP-K^{Sim4} are required for CENP-A nucleosome deposition (Fig. 3) (Pidoux et al. 2009; Takahashi et al. 2000). Similar to CENP-C, tethering CENP-I to a non-centromeric site in chicken DT40 cells drives new CENP-A deposition and forms an epigenetic centromere (Hori et al. 2013). This suggests that the CCAN components play a dual role, and are required for both centromere specification in G1 and recruitment of kinetochore components during mitosis.

Budding yeast centromeres are determined by DNA sequence. And although they share a homologous CENP-A chaperone, Scm3, the mechanism by which Scm3 is recruited to centromeres is distinct from epigenetic centromeres (Fig. 3). The centromere-determining elements (CDE) in budding yeast are essential for recruitment of a DNA-binding protein Cbf1 specifically recognizing CDEI and a multisubunit protein complex: CBF3 (centromere

binding factor 3), containing Ndc10, Cep3, Ctf13, and Skp1, associated with CDEIII DNA element (Cho and Harrison 2011; Doheny et al. 1993; Goh and Kilmartin 1993; Hyman et al. 1992; Lechner and Carbon 1991; Mizuguchi et al. 2007; Russell et al. 1999; Strunnikov et al. 1995). The CBF3 subunit-Ndc10 is required for the recruitment of the Scm3 chaperone and subsequent deposition of the CENP-A^{Cse4} containing nucleosome (Camahort et al. 2007; Mizuguchi et al. 2007).

4 The Chromatin Landscape Influence on CENP-A Deposition

CENP-A nucleosomes are interspersed with the canonical H3 nucleosomes within the centromeres of flies and humans (Blower et al. 2002). Centromeres were initially thought to be transcriptionally silent loci, a characteristic that is consistent with the posttranslational modifications found in the surrounding pericentric heterochromatin (Peters et al. 2001; Ribeiro et al. 2010; Rice et al. 2003). However, studies in human and *Drosophila*-derived chromatin fibers demonstrated that H3K9me2 and H3K9me3 marks are absent from the CENP-A (CID) domain (Lam et al. 2006; Sullivan and Karpen 2004). Furthermore, histone H3 nucleosomes found interspersed with CENP-A nucleosomes in humans are decorated with histone marks associated with active or poised chromatin, such as H3K4me1/2 and H3K36me2/3 (Bergmann et al. 2011; Sullivan and Karpen 2004). The histone H3K4 trimethylation, associated with actively transcribed regions, is absent from the centromeric core domain in humans and *Drosophila*, but is present at chicken centromeric DNA (Ribeiro et al. 2010; Sullivan and Karpen 2004). Until recently the centrochromatin-localized histones in higher eukaryotes were thought to be hypo-acetylated and lack acetylated marks found generally in euchromatin such as H3K9Ac, H4K5Ac, H4K8Ac, H4K12Ac, or H4K16Ac. However, a recent study documented the presence of H4K5Ac and H4K12Ac within CENP-A containing nucleosomes in chicken and humans (Bailey et al. 2016; Shang et al. 2016; Sullivan and Karpen 2004).

Transcripts from centromeric repeat sequences have been observed in multiple model organisms (Bergmann et al. 2011; Bouzinba-Segard et al. 2006; Carone et al. 2009, 2013; Chan et al. 2011, 2012; Eymery et al. 2009; Hall et al. 2012; Lam et al. 2006; May et al. 2005; Ohkuni and Kitagawa 2011; Quenet and Dalal 2014b; Stimpson and Sullivan 2010; Topp et al. 2004; Wong et al. 2007). Active RNA Polymerase II is recruited to endogenous human centromeres during mitosis and early G1 (Chan et al. 2012; Quenet and Dalal 2014b). Inhibition of RNA-Polymerase-II-mediated transcription in HeLa cells leads to decreased α -satellite transcript levels in mitosis, loss of CENP-C recruitment to endogenous centromeres, and chromosome segregation defects (Chan et al. 2012). The mechanistic role of centromeric transcripts and the act of transcription in centromere function is not yet clear, although histone H3 eviction may be a key aspect.

Utilizing a synthetic human artificial chromosome (HAC), Bergmann et al. demonstrated that the presence of H3K4me2 and transcription events at the centromere play a critical role in CENP-A assembly and centromere function by altering the recruitment of CENP-A deposition machinery (Bergmann et al. 2011). Tethering a lysine-specific demethylase 1 (LSD1) to the HAC centromeric domain leads to removal of H3K4 methylation and results in loss of transcription of α -satellite DNA at this loci. This correlates with loss of HJURP

localization, impaired CENP-A deposition, and ultimately leads to loss of kinetochore function (Bergmann et al. 2011).

Biochemical purification of RNA associated with the prenucleosomal CENP-A/HJURP complex identified a 1.3 kb RNA product that co-localizes with α -satellite DNA and CENP-A, and hybridizes to centromeric α -satellite probes, suggesting it originated from α -satellite transcripts (Quenet and Dalal 2014a). Targeting of α -satellite transcripts as well as other centromere-derived RNAs by siRNA in vivo results in reduced CENP-A and HJURP recruitment to the centromere, suggesting that the RNA component partially encoded within α -satellite DNA plays a role in CENP-A deposition pathway (Quenet and Dalal 2014a). Exactly how RNAs are associated with the CENP-A prenucleosomal complex is still unknown, as well as how this association would contribute mechanistically to CENP-A deposition.

A strong link between CENP-A deposition and transcription was demonstrated in *Drosophila*. Chen et al. using an inducible ectopic centromere approach demonstrated that new CENP-A^{CID} deposition at the ectopic centromere requires transcription (Fig. 3) (Chen et al. 2015). The mass spec analysis of binding partners of the *Drosophila* CENP-A^{CID} chaperone CAL1 in vivo identified two subunits of the FACT complex: Spt16 and SSRP1, both of which physically interact with CAL1. FACT was also previously found associated with centromere in human cells (Foltz et al. 2009; Obuse et al. 2004). FACT is involved in transcription elongation from chromatin templates in vitro and promoting deposition of histone H3.3 nucleosomes in vivo in *Drosophila* (Orphanides et al. 1998). Spt16 and SSRP1 subunits colocalize with CENP-A^{CID} in *Drosophila* cells and downregulation of FACT leads to defects in CENP-A^{CID} recruitment at endogenous centromeres. CAL1 along with FACT facilitates RNA-Polymerase-II-mediated transcription at the site of CENP-A^{CID} deposition, which is required for CENP-A^{CID} incorporation to occur. In support to these findings other groups reported localization of the active form of RNA Polymerase II at endogenous centromeres in *Drosophila* during mitosis, which is coincident with new CENP-A^{CID} deposition timing (Rosic et al. 2014).

In addition to the role of the Mis18 complex in the recognition of the CCAN and direct recruitment of HJURP, the Mis18 complex influences posttranslational modifications within the centromeric chromatin (Kim et al. 2012). Deletion of Mis18 in *S. pombe* leads to increased levels of histone H3 and H4 acetylation at centromeres (Hayashi et al. 2004). In vertebrates, the Mis18 complex influences histone modifications and DNA methylation. Knockout of Mis18 α in mice leads to reduced H3K9 and H3K4 methylation and increased acetylation within centromeric repeats (Kim et al. 2012). The de novo methyltransferase enzymes DNMT3a/b are also recruited to centromeres by Mis18 α/β (Kim et al. 2012). Downregulation of DNMT3b or Mis18 α leads to increased transcription of centromeric repeats (Gopalakrishnan et al. 2009). However, the importance of DNMT3a/b in centromere function is unclear since cells lacking DNMT3a/b are viable (Reviewed in Brown and Robertson 2007).

More recently Mis18BP1 was shown to recruit the KAT7 lysine methyltransferase complex to centromeres (Ohzeki et al. 2016). Disruption of the KAT7 complex leads to reduced

CENP-A deposition. KAT7 in conjunction with RSF1 may regulate histone turnover to facilitate new CENP-A deposition in G1. In future work it will be important to determine exactly how the Mis18 complex may integrate multiple downstream chromatin modifying pathways to promote centromere deposition.

5 Licensing of Centromere Assembly

CENP-A incorporation into the centromeric chromatin is cell cycle regulated, although the timing of CENP-A deposition differs across species (Figs. 1 and 4) (Allshire and Karpen 2008; Boyarchuk et al. 2011). Budding yeast CENP-A^{Cse4} incorporation is coincident with DNA replication (Pearson et al. 2004; Wisniewski et al. 2014). Similarly, in fission yeast, CENP-A deposition occurs during early S phase, but also during G2 phase (Takayama et al. 2008). In vertebrates, new CENP-A incorporation is uncoupled from DNA replication and restricted to late telophase/early G1 phase (Bernad et al. 2011; Jansen et al. 2007; Silva et al. 2012).

The process of human CENP-A deposition occurs via a licensing mechanism that restricts deposition to the G1 phase and controls the assembly of CENP-A to ensure that only a limited amount of new CENP-A is assembled in each cell cycle. The timing of CENP-A deposition is restricted to the early G1 phase by inhibition of CENP-A deposition through CDK activity, which is high during S and G2-phase, and drops rapidly following satisfaction of the mitotic checkpoint (Fig. 1) (Silva et al. 2012). Although CENP-A transcript and protein levels accumulate from mid-S phase into G2, CDK1/CDK2-dependent phosphorylation of Mis18BP1 prevents from premature CENP-A loading during this time (Silva et al. 2012). Mis18BP1 dephosphorylation occurs during early G1, coincident with new CENP-A deposition. The PLK1 kinase positively regulates CENP-A deposition. PLK1 phosphorylates the Mis18 complex during G1 to promote its recruitment to centromeres (Fig. 1) (McKinley and Cheeseman 2014). Inhibition of the PLK1 kinase activity abrogates new CENP-A deposition. The opposing functions of PLK1 and CDK1 phosphorylation provide tight temporal control of CENP-A deposition by limiting Mis18 recruitment.

The assembly of CENP-A nucleosomes in G1 is limited by at least two mechanisms. The Mis18 complex forms a conserved multimer (Nardi et al. 2016; Subramanian et al. 2016). Mis18 binds the centromere stably in late telophase. Binding of HJURP to Mis18 disrupts the Mis18 multimer and eliminates the ability of Mis18 to continue to interact with the centromere, essentially removing the signal for HJURP recruitment, and blocking further CENP-A deposition at that site. In addition, the Mis18 β subunit undergoes ubiquitylation and degradation by the SCF ^{β TrCP} E3 ubiquitin ligase, thus degrading the signal for HJURP recruitment to centromeres (Kim et al. 2014).

6 Viva la Difference—Evolutionary Diversity in CENP-A Deposition Pathways

Despite the high degree of conservation between the CENP-A-binding domains within the HJURP and Scm3 chaperones, that spans billions of years of evolution, there is a great variety in the CENP-A deposition pathways across organisms (Figs. 3 and 4). This likely

reflects the unique strategies for centromeric chromatin assembly that these organisms employ.

Drosophila species lack a clear HJURP homolog, but an siRNA screen for genes involved in CENP-A^{CID} centromere deposition in *Drosophila* S2 cells identified CAL1 (chromosome alignment defect 1) as a key factor (Erhardt et al. 2008). *Drosophila* CAL1 is a fly-specific protein that functions as a CENP-A^{CID} chaperone (Chen et al. 2014; Erhardt et al. 2008; Goshima et al. 2007). Despite the small similarity to the Scm3 domain of *K. lactis*, based on sequence and secondary structure, CAL1 does not share common ancestry with yeast Scm3 and human HJURP (Phansalkar et al. 2012; Sanchez-Pulido et al. 2009). CAL1 directly binds to CENP-A^{CID}/H4 dimer and was shown to function as the CENP-A^{CID}-specific assembly factor in fruit flies (Chen et al. 2014). Its depletion in *Drosophila* results in loss of centromeric CENP-A^{CID} localization and is associated with chromosome segregation defects (Chen et al. 2014; Erhardt et al. 2008; Goshima et al. 2007). Both HJURP and CAL1 are sufficient to promote de novo centromere establishment. Tethering HJURP to the chromosome arm or to a naïve alpha satellite array is sufficient to facilitate CENP-A deposition outside of the centromeric chromatin and results in formation of a functional kinetochore at an ectopic site in human cells (Barnhart et al. 2011; Ohzeki et al. 2012). Similarly, targeting CAL1 to an ectopic site was demonstrated to mediate de novo CENP-A^{CID} deposition in *Drosophila*, which leads to formation of a de novo centromere outside of the endogenous centromeric loci (Chen et al. 2014). This de novo centromere is epigenetically maintained and serves as platform for recruitment of a functional kinetochore (Chen et al. 2014). There are several organisms that contain CENP-A nucleosomes for which a functional chaperone has not been identified, including the well-studied nematode *C. elegans* (Fig. 4). *C. elegans* have holocentric chromosomes in which the centromere position may be variable and obfuscate the need for specific targeting of the CENP-A histone variant.

Conservation of the Mis18 complex is also highly variable across species. Species as divergent as *S. pombe* and humans possess Mis18, but in higher eukaryotes the Mis18 gene underwent duplication (Fig. 4). The Mis18 paralogs, termed Mis18 α and Mis18 β , share about 30% sequence identity, but have diverged in their function in higher eukaryote centromeres (Fujita et al. 2007; Hayashi et al. 2004; Stellfox et al. 2016). The Mis18 complex has not been identified in *Drosophila* or *S. cerevisiae* (Fig. 4). In both cases, these organisms have devised alternative strategies to couple the CENP-A chaperones to the existing centromere. CAL1 binds CENP-C in *Drosophila* and the Ndc10 complex, which directly recognizes DNA, recruits the Scm3 chaperone in budding yeast (Camahort et al. 2007; Doheny et al. 1993; Erhardt et al. 2008; Goh and Kilmartin 1993; Jiang et al. 1993; Lechner and Carbon 1991; Mellone et al. 2011; Sorger et al. 1995).

While *S. pombe* possess a Mis18 homolog, it lacks the vertebrate Mis18BP1 orthologue (Fig. 4). The Mis18BP1 function in *S. pombe* is replaced by the Eic1 protein (a.k.a Mis19) (Fig. 3). The Eic1 and Eic2 proteins co-purified with the spMis18 and exhibit a similar temporal pattern of centromeric localization throughout the cell cycle (Hayashi et al. 2014; Subramanian et al. 2014). Eic1 was demonstrated to be essential for the recruitment of the Mis18, Mis16, and Scm3 proteins to the centromere and for CENP-A^{Cnp1} incorporation.

However, Eic2 is dispensable for recruitment of CENP-A^{Cnp1} to the centromere. This suggests Eic1 is functionally analogous to the Mis18BP1 subunit in recruitment of CENP-A deposition, although Eic1 is evolutionary distinct and does not share any apparent sequence homology to Mis18BP1 (Hayashi et al. 2014; Subramanian et al. 2014).

7 Centromere Stabilization and Re-organization

The recruitment of CENP-A to centromeres via HJURP and Mis18 is not sufficient for the stability of CENP-A, but requires additional proteins that may potentially reorganize centromeric chromatin to increase stability. These factors include the Rho GTPase MgcRacGAP, the formin protein mDia, and the RSF-1 remodeling complex, and appear to be recruited to centromeres later than Mis18 and HJURP (Fig. 1) (Izuta et al. 2006; Lagana et al. 2010; Liu and Mao 2016; Obuse et al. 2004; Perpelescu et al. 2009).

MgcRacGAP co-purifies with centromeric chromatin and with Mis18BP1 from HeLa cells (Izuta et al. 2006; Lagana et al. 2010; Perpelescu et al. 2009). MgcRacGAP localizes to centromeres in late G1. Although the exact timing between MgcRacGAP recruitment and HJURP recruitment has not been established, it appears that MgcRacGAP is recruited later, after new CENP-A incorporation is accomplished. Depletion of MgcRacGAP or its binding partner, ECT2 (guanine nucleotide exchange factor), results in loss of newly incorporated CENP-A, while existing CENP-A is not affected. This suggests that new and old CENP-A populations during G1 are in some way unique. Furthermore, Cdc42, a small GTPase identified as a target of MgcRacGAP-ECT2 complex, is also recruited to the centromeres during interphase. The Cdc42 activity requires GTPase cycling mediated by MgcRacGAP-ECT2, proposing a GTPase switch implicated in the maturation of the newly deposited CENP-A containing nucleosomes (Lagana et al. 2010). mDia2 is a downstream effector of Rho signaling (Gasman et al. 2003; Lammers et al. 2008). mDia2 depletion leads to defects in new CENP-A deposition. The constitutively active form of mDia2 restores CENP-A levels at the centromere resulting from MgcRacGAP downregulation, consistent with its role downstream of MgcRacGAP in this process. Interestingly, mDia2 depletion leads to prolonged HJURP association with the centromere, suggesting that the processes of HJURP recruitment and MgcRacGAP stabilization are mechanistically linked (Liu and Mao 2016).

The RSF (remodeling and spacing factor), comprised the RSF-1 and SNF2h subunits, has been characterized as an ATP-dependent nucleosome remodeling and spacing factor that together with the FACT complex is implicated in transcription initiation (LeRoy et al. 1998; Orphanides et al. 1998). The RSF complex co-purified with CENP-A nucleosomes prepared from interphase cell extracts (Izuta et al. 2006; Obuse et al. 2004; Perpelescu et al. 2009). RSF centromere localization peaks during the middle of G1 phase. RSF1 can reconstitute and space CENP-A nucleosomes on a naked DNA template, and is required for stability of CENP-A nucleosomes within the centromeric chromatin (Perpelescu et al. 2009). This argues that energy-dependent remodeling events are involved in stabilization of newly deposited CENP-A nucleosomes.

Condensation of centromeric chromatin is a potentially important step in efficient CENP-A deposition. The condensin complexes are involved in ATP-dependent chromosome

condensation during mitosis, and are also implicated in centromere establishment in yeast and humans (Hagstrom et al. 2002; Ono et al. 2004; Samoshkin et al. 2009; Wignall et al. 2003; Yong-Gonzalez et al. 2007). Of the two partially overlapping condensin complexes that have been characterized (Condensin I and II) the Condensin II complex is selectively involved in centromeric chromatin assembly (Barnhart-Dailey et al. 2016; Bernad et al. 2011; Hirano 2005). Downregulation of common components to the Condensin complexes (SMC2 and SMC4) or the Condensin-II-specific subunits (Caph2 and CapD3) leads to reduced assembly of new CENP-A nucleosomes in humans and *Xenopus* extracts (Barnhart-Dailey et al. 2016; Bernad et al. 2011; Samoshkin et al. 2009). CAPH2 was found at human centromeres in early G1, coincident with new CENP-A deposition, and its recruitment is HJURP dependent (Fig. 1) (Barnhart-Dailey et al. 2016).

In chicken cells FACT subunits: SSRP1 and SPT16 co-purified with CENP-A and localize to the centromeric chromatin. FACT interacts with ATP-dependent chromatin remodeling factor CHD1, and the centromeric recruitment of these proteins throughout the cell cycle is dependent upon the CENP-HIKM complex. The downregulation of FACT or CHD1 factors leads to loss of new CENP-A deposition, demonstrating that chromatin remodeling activity of the FACT and CHD1 complexes plays a critical role in CENP-A deposition (Okada et al. 2009). It remains elusive whether the FACT and CHD1 complexes require active transcription in order to fulfill their role in CENP-A incorporation.

8 HJURP, CENP-A, and Cancer

Coordinated up-regulated expression of CENP-A and HJURP mRNA is observed in many cancers, including breast cancer, adenocarcinoma of the colon, gliomas and lipomas, and is a potentially powerful biomarker in several distinct types of cancers (Athwal et al. 2015; Dai et al. 2012; de Tayrac et al. 2011; Hu et al. 2010; Tomonaga et al. 2003; Valente et al. 2013; Wang et al. 2009). Misregulation of CENP-A deposition is a potential mechanism of generating genome instability, as even the missegregation of a single chromosome is sufficient to cause the rearrangement of that chromosome in a process called chromothripsis (Stephens et al. 2011; Zhang et al. 2015). This co-overexpression may be driven by the dysregulation of the transcription factor FoxM1 (Thiru et al. 2014); however, phenotypic consequences of CENP-A overexpression are beginning to emerge. Elevated CENP-A levels in human cells and other organisms result in mistargeting of CENP-A to ectopic sites and can lead to genomic instability (Choi et al. 2011, 2012; Gascoigne et al. 2011; Heun et al. 2006; Mendiburo et al. 2011; Mishra et al. 2011; Van Hooser et al. 2001). Overexpressed CENP-A can form a heterotypic nucleosome containing one copy of CENP-A and one copy of H3.3. The accumulation of CENP-A in the chromosome arms occurs by co-opting the H3.3 chaperone DAXX to mediate its mislocalization (Lacoste et al. 2014).

In budding yeast, the misregulation of CENP-A^{Cse4} proteolysis results in accumulation of CENP-A^{Cse4} in gene promoters and causes altered gene expression (Hildebrand and Biggins 2016). Accumulation of CENP-A overexpressed in human cells has been observed in CTCF sites, DNaseI hypersensitive sites, and regions of nucleosome turnover, as well as at some key oncogene promoters (Athwal et al. 2015; Lacoste et al. 2014). The question remains whether the overexpression of CENP-A will drive genomic instability in tumors, and if so

whether the presence of CENP-A at the ectopic sites alters the transcriptional profile of underlying genes, changes 3D chromatin arrangement due to disruption of CTCF sites, or weakens of endogenous centromere by the redistribution of centromere components is the key event.

9 Conclusions

Centromere specification is essential to ensure genome stability, as defects in centromere establishment can lead to errors in chromosome segregation during mitosis. Because centromeres of many higher eukaryotes are specified epigenetically by the presence of a unique nucleosome containing the histone variant CENP-A, the key step in centromere inheritance is the assembly of new CENP-A nucleosomes. Diverse organisms with unique strategies for centromere inheritance utilize the recruitment of a CENP-A-specific chaperone to ensure the perpetuation of centromere identity. In humans, the centromere deposition machinery is coupled to centromeric proteins that depend on CENP-A for their localization, thus creating an epigenetic mechanism for inheritance of the centromeric locus.

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Fig. 1.

The model of cell cycle regulated CENP-A deposition in humans. New CENP-A deposition occurs exclusively during early G1 and protein complexes involved are depicted in the model. HJURP binds the newly synthesized CENP-A/H4 complex in pre-nucleosomal form (Dunleavy et al. 2009; Foltz et al. 2009; Shuaib et al. 2010). H4K5Ac and H4K12Ac histone marks present in the CENP-A pre-nucleosomal complex are dependent upon RbAp46/48/HAT1 activity and required for CENP-A deposition (Shang et al. 2016). HJURP/CENP-A/H4 localization relies on the Mis18 complex (Barnhart et al. 2011; Fujita et al. 2007). The CENP-A deposition machinery is controlled by CDK activity. Cell cycle regulated and CDK1/CDK2-dependent phosphorylation of Mis18BP1 and HJURP prevents premature CENP-A loading during G2 and mitosis, and dephosphorylation of these proteins occurs prior new CENP-A deposition in G1 (Muller et al. 2014; Silva et al. 2012). G1-coupled and PLK1-mediated phosphorylation of the Mis18 complex promotes its centromeric localization and CENP-A deposition (McKinley and Cheeseman 2014). Mis18BP1 is recruited to centromeres upon its direct interaction with CENP-C (Dambacher et al. 2012). Human Mis18 α and Mis18 β form a multi subunit complex which is recruited to the centromere through interaction of Mis18 α with Mis18BP1 and Mis18 β with CENP-C (Nardi et al. 2016; Stellfox et al. 2016). HJURP mediates deposition of CENP-A nucleosomes, and histone H3.3 placeholder is removed from the centromeric chromatin (Dunleavy et al. 2011). Following new CENP-A deposition centromeric nucleosomes are stabilized and protein factors involved in this process are depicted in the model. During DNA replication existing CENP-A nucleosomes are retained across the replication fork (Bodor et al. 2014; Jansen et al. 2007)

Fig. 2.

Molecular organization of CENP-A-specific chaperone in different species. Domains identified within CENP-A chaperones among different species and their roles are depicted (Barnhart et al. 2011; Bassett et al. 2012; Cho and Harrison 2011; Dechassa et al. 2011; Hu et al. 2011; Muller et al. 2014; Sanchez-Pulido et al. 2009; Schittenhelm et al. 2010; Shuaib et al. 2010; Wang et al. 2014; Zasadzinska et al. 2013). The Scm3 domain is conserved among eukaryotes except for the *Drosophila melanogaster* where the similarity was assessed based on both sequence and secondary structure similarity (Phansalkar et al. 2012; Sanchez-Pulido et al. 2009). HMD-HJURP mid domain; HCTD1-HJURP carboxy terminal domain 1; HCTD2-HJURP carboxy terminal domain 2

Fig. 3.

Protein complexes involved in CENP-A deposition pathway in eukaryotes. The comparison of CENP-A deposition machinery across species. All conserved proteins involved in the CENP-A deposition pathway are colored similarly. Budding yeast point centromeres are specified by unique DNA elements: CDEI, CDEII, and CDEIII (Clarke and Carbon 1980; Fitzgerald-Hayes et al. 1982), which are required for recruitment of DNA-binding proteins as depicted in the model. The regional centromeres in fission yeast and higher eukaryotes are specified by the presence of CENP-A containing nucleosomes. CENP-A incorporation into centromeric chromatin is mediated by its distinct histone chaperone HJURP in vertebrates, Scm3 in yeast and CAL1 in *Drosophila melanogaster* (Barnhart et al. 2011; Bernad et al. 2011; Camahort et al. 2007; Dechassa et al. 2011; Dunleavy et al. 2009; Foltz et al. 2009; Mizuguchi et al. 2007; Pidoux et al. 2009; Shuaib et al. 2010; Stoler et al. 2007; Williams et al. 2009). HJURP and Scm3 share common ancestry, as depicted on the model, and CAL1 shares similarity to Scm3 based on the sequence and secondary structure similarity (Phansalkar et al. 2012; Sanchez-Pulido et al. 2009). CENP-C is conserved in all eukaryotes but its essential role in centromere specification is restricted to higher eukaryotes where it is required for recruitment of the Mis18 complex (Dambacher et al. 2012; Moree et al. 2011). The role of the Mis18 complex in CENP-A deposition pathway is conserved from

fission yeast to humans; however, no Mis18 homologue was identified in *Drosophila* (Fujita et al. 2007; Maddox et al. 2007). The fission yeast has only one copy of Mis18 protein and the function of Mis 8BP1 was replaced by the Eic1 protein (Hayashi et al. 2014; Subramanian et al. 2014). Human Mis18 complex is a multisubunit complex composing of Mis18 α/β and Mis18BP1 (Fujita et al. 2007; Maddox et al. 2007; Nardi et al. 2016). CENP-A deposition in *Drosophila* requires active transcription mediated by the FACT and RNA Polymerase II (Chen et al. 2015)

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Species	Mis18	Mis18BP1/ counterpart	CENP-A chaperone	CENP-C	CENP-A deposition timing
<i>S. cerevisiae</i>	Not Identified	Not Identified			S phase
<i>S. pombe</i>					S phase/ G2
<i>Ustilago</i>		Not Identified	Not Identified		Unknown
<i>C. elegans</i>	Not Identified		Not Identified		Mitosis
<i>Drosophila</i>	Not Identified	Not Identified			Metaphase/ Anaphase
<i>Zebrafish</i>					Unknown
<i>Xenopus</i>					Early Interphase
<i>Mouse</i>					Unknown
<i>Human</i>					Late telophase/ early G1

Fig. 4. Conservation of CENP-A deposition factors across species. Table detailing the conserved proteins involved in CENP-A deposition pathway as well as timing of CENP-A deposition in different model organisms (Bernad et al. 2011; Dunleavy et al. 2007; Jansen et al. 2007; Maddox et al. 2007; Mellone et al. 2011; Moree et al.2011; Pearson et al. 2004; Schuh et al. 2007; Takayama et al. 2008)