25. Tomancak P, Guichet A, Zavorszky P, Ephrussi A (1998) Oocyte polarity depends on regulation of gurken by Vasa. Development 125: 1723–1732.
26. King RC (1970) Ovarian development in *Drosophila melanogaster*. New York: Academic Press. 227 p.
27. Parfenov V, Potchukalina G, Dudina L, Kostyuchek D, Gruzova M (1988) Human antral follicles: oocyte nucleus and the karyosphere formation (electron microscopic and autoradiographic data). Gamete Res 22: 219–231.
28. Ivanovska I, Khandan T, Ito T, Orr-Weaver TL (2005) A histone code in meiosis: the histone kinase, NHK-1, is required for proper chromosomal architecture in *Drosophila* oocytes. Genes Dev 19: 2571–2582.
29. Cullen CF, Brittle AL, Ito T, Ohkura H (2005) The conserved kinase NHK-1 is essential for mitotic progression and unifying acentrosomal meiotic spindles in *Drosophila melanogaster*. J Cell Biol 171: 593–602.
30. Aihara H, Nakagawa T, Yasui K, Ohta T, Hirose S, et al. (2004) Nucleosomal histone kinase-1 phosphorylates H2A Thr 119 during mitosis in the early *Drosophila* embryo. Genes Dev 18: 877–888.
31. Lancaster OM, Cullen CF, Ohkura H (2007) NHK-1 phosphorylates BAF to allow karyosome formation in the *Drosophila* oocyte nucleus. J Cell Biol 179: 817–824.
32. Riechmann V, Ephrussi A (2001) Axis formation during *Drosophila* oogenesis. Curr Opin Genet Dev 11: 374–383.
33. Harrison JC, Haber JE (2006) Surviving the breakup: the DNA damage checkpoint. Annu Rev Genet 40: 209–235.
34. Page SL, Hawley RS (2001) *c(3)G* encodes a *Drosophila* synaptonemal complex protein. Genes Dev 15: 3130–3143.
35. Savvidou E, Cobbe N, Steffensen S, Cotterill S, Heck MM (2005) *Drosophila* CAP-D2 is required for condensin complex stability and resolution of sister chromatids. J Cell Sci 118: 2529–2543.
36. Resnick TD, Dej KJ, Xiang Y, Hawley RS, Ahn C, et al. (2009) Mutations in the chromosomal passenger complex and the condensin complex differentially affect synaptonemal complex disassembly and metaphase I configuration in *Drosophila* female meiosis. Genetics 181: 875–887.
37. Ashburner M, Golic KG, Hawley RS (2005) *Drosophila*: a laboratory handbook. New York: Cold Spring Harbor Laboratory Press. 1409 p.
38. Drysdale RA, Crosby MA, The FlyBase Consortium (2005) FlyBase: genes and gene models. Nuc Acids Res 33: D390–D395.
39. Lindsley DL, Zimm GG (1992) The genome of *Drosophila malanogaster*. New York: Academic Press. 1133 p.
40. Lasko PF, Ashburner M (1990) Posterior localization of *vasa* protein correlates with, but is not sufficient for, pole cell development. Genes Dev 4: 905–921.
41. Harlow E, Lane D (1988) Antibodies: a laboratory manual. New York: Cold Spring Harbor Laboratory Press. 726 p.
42. Theurkauf WE, Smiley S, Wong ML, Alberts BM (1992) Reorganization of the cytoskeleton during *Drosophila* oogenesis: implications for axis specification and intercellular transport. Development 115: 923–936.
43. Stuurman N, Maus N, Fisher PA (1995) Interphase phosphorylation of the *Drosophila* nuclear lamin: site-mapping using a monoclonal antibody. J Cell Sci 108: 3137–3144.
44. Anderson LK, Royer SM, Page SL, McKim KS, Lai A, et al. (2005) Juxtaposition of C(2)M and the transverse filament protein C(3)G within the central region of *Drosophila* synaptonemal complex. Proc Natl Acad Sci U S A 102: 4482–4487.
45. Brittle AL, Ohkura H (2005) Mini spindles, the XMAP215 homologue, suppresses pausing of interphase microtubules in *Drosophila*. EMBO J 24: 1387–1396.

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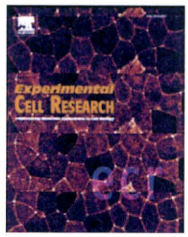

Review

Core histone H2A ubiquitylation and transcriptional regulation

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ABSTRACT

Diverse histone modifications, such as acetylation, methylation and ubiquitylation have been linked to the regulation of cellular activities such as transcription, repair and replication. The mechanisms by which histone modifications contribute to the transcription process are not fully understood; however increasing evidence suggests that they work together in the form of a histone code to regulate the recruitment of chromatin-modulating factors [1–3]. Histone ubiquitylation has been found to be an important chromatin modification with roles in trans-histone modification and transcriptional regulation. In the past several years, there has been dramatic progress in the identification of factors that control ubiquitin attachment to the histone. Recent advances concerning core histone H2A ubiquitylation and transcriptional regulation will be reviewed and the cellular functions of these histone modifications will be discussed.

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In the eukaryotic nucleus, genomic DNA interacts with numerous proteins to form chromatin. Packaging of the template into chromatin appears to affect all stages of transcription, including initiation and elongation. Eukaryotic gene expression is regulated by chromatin structure together with the cellular network of cis-acting elements and trans-acting factors (for reviews [4,5]. The nucleosome is the fundamental unit of chromatin and is composed of 147 base pairs (bp) of DNA wrapped 1.65 turns around the histone octamer of the four core histones (H2A, H2B, H3, and H4) [6]. Nucleosomes act as general repressors of multiple stages of transcription, including initiation, promoter clearance, elongation, and termination. According to recent concepts of the histone code, post-translational modifications

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of the histone tail represent a major mechanism by which cells control the structure and function of chromatin [3,7,8]. Among diverse histone modifications, histone ubiquitylation has emerged as an important chromatin modification with roles in transcriptional regulation.

Histone H2A ubiquitylation

Ubiquitylation is composed of the reactions of the 76-amino-acid-residue ubiquitin protein upon activation and is conjugated to target proteins by an isopeptide bond between the carboxyl terminal Glycine of ubiquitin and the Lysine of the target protein or of ubiquitin itself. Ubiquitin ligation to protein requires the sequential action of three enzymes. The carboxyl terminal Glycine 76 residue of ubiquitin is activated in an ATP-dependent fashion by the activating enzyme, E1. Ubiquitin adenylate is formed by release of PPi and its subsequent hydrolysis, followed by the binding of ubiquitin to a Cys residue of E1 in a thiolester linkage. Then, activated ubiquitin is transferred to a Cys residue of the ubiquitin-carrier protein E2. In the last step of ubiquitylation, the carboxyl terminal Glycine of ubiquitin is linked to the ε-amino group of a Lys residue of the substrate by an isopeptide linkage using the ubiquitin-protein ligase E3 enzyme. There is usually a single E1 and there are more E2 proteins and multiple specific E3 proteins. Each E3 protein appears to be responsible for the specific ubiquitin-protein ligation [9].

More than three decades have passed since the discovery that the core histone H2A is monoubiquitylated and its ubiquitylation changes during hepatocyte regeneration. Histone H2A was the first protein identified as being ubiquitylated [10], and the ubiquitylation site has been mapped to the highly conserved residue Lys 119 [11].

Although early studies suggested the involvement of H2A ubiquitylation in gene activation [12,13], recent studies have indicated a role for H2A ubiquitylation in gene repression [14–17]. About 10% of total H2A has been reported to be ubiquitylated in higher eukaryotic organisms, however ubiquitylated H2A (uH2A) has not been reported in the budding yeast [18].

On the other hand, ubiquitylated histone H2B was discovered in 1980 [19] and it was clarified that ubiquitin is conjugated to Lysine 120 of mammalian H2B or to Lysine 123 of yeast H2B in the C-terminus of H2B [18,20]. The ubiquitin moiety on H2B is dynamically regulated during gene expression in yeast, turning over at promoters and coding regions during both initiation and elongation phases of transcription [21–23].

Polyubiquitylated proteins are usually marked for degradation by the 26S proteosome. In contrast, monoubiquitylated proteins are stable [24,25] and the cellular fate of these proteins is very different from that of polyubiquitylated proteins [26]. Histones H2A and H2B are monoubiquitylated and ubiquitylated H2A (uH2A) typically represents 10% of H2A, and ubiquitylated H2B (uH2B) represents 1–2% of H2B [19,27,28].

The H2A ubiquitylation site is located on the H2A docking domain (amino acids 80–119) which accounts for the accessible surface area upon binding of a H2A–H2B dimer to the H3–H4 tetramer. A section of the H2A C-terminal tail runs antiparallel which further links it to the underlying H3–H4 histone-fold domains [6]. Lysine 119 is found at the end of the docking domain and at the beginning of the C-terminal tail of uH2A in the trypsin accessible region of H2A [29]. Analysis of crystal structure showed that Lysine 119 is located on the surface of the histone octamer [6]. Thus, histone H2A Lysine 119 is potentially accessible by the ubiquitin-conjugating or deubiquitylating machinery and it can be assumed that monoubiquitylation of histone H2A on Lysine 119 affects the bulk configuration of the nucleosome. However, change of bulk configuration of the nucleosome was not detected by *in vitro* nucleosomal assembly assay using ubiquitylated H2A [14]. It was also reported that ubiquitylated H2A did not prevent binding of histone H1 but rather enhanced the binding of this histone to the nucleosome [30].

As monoubiquitylation of histone H2A on Lysine 119 in its C-terminal tail has been found in higher eukaryotes but not in Saccharomyces cerevisiae [29,31], histone H2A ubiquitylation is not essential for cell growth, sporulation, or resistance to either heat stress or UV radiation as seen in S. cerevisiae but is necessary for higher order functions seen only in higher eukaryotes.

Histone H2A ubiquitin ligase

The enzyme responsible for H2A ubiquitylation is found by purification and functional characterization of an E3 ubiquitin ligase activity specific for histone H2A [32]. Human Polycomb repressive complex 1-like protein (hPRC1L) is composed of several Polycomb-group proteins including Ring1, Ring2 and Bmi1 (Ring1A, Ring 1B and Bmi1 in the mouse, respectively). hPRC1L monoubiquitylates histone H2A Lysine 119 in the nucleosome form *in vitro*. It was found that Ring2 contains an intrinsic E3 ligase activity and that the conserved Arg 70 in the RING domain of Ring2 is critical for the enzymatic activity. Knock down of Ring2 decreases the level of ubiquitylated H2A resulting in changes of cellular morphology and slower cell growth *in vivo*. In addition using Drosophila wing imaginal discs it was shown a dRing colocalization with ubiquitylated H2A at Polycomb response element (PRE) and promoter regions of the Ubx gene. It was also shown that removal of dRing by RNA interference of SL2 tissue culture cell resulted in loss of H2A ubiquitylation. Thus it was suggested that the hRing2 and dRing are H2A ubiquitin ligases and relate to Polycomb silencing [32]. It has also been found that 2A-HUB/hRUL138 is a histone H2A ubiquitin ligase. 2A-HUB was identified by its association with the N-CoR/HDAC1/3 complex that is recruited to a subset of regulated gene promoters. 2A-HUB catalyzes monoubiquitylation of H2A and is reported to function as a component of the repression machinery required for specific gene regulation. 2A-HUB is reported to mediate repression of chemokines related to TLR activation. It is suspected that distinct H2A ubiquitin ligases each recruited based on interactions with different corepressor complexes, contribute to distinct transcriptional repression programs [33].

Two ubiquitin ligases Ring2/Ring1b and 2A-HUB as transcriptional regulators support the concept that H2A ubiquitylation is associated with transcriptional repression. As E3 ligase RNF8 catalyzes ubiquitylation of H2A as well as that of H2AX to maintain genome stability [34–36] it suggests an additional role for H2A ubiquitylation in DNA repair. Cell responses to DNA double-strand breaks (DSBs) utilize post-translational modifiers to regulate cell-cycle checkpoints, DNA repair, cellular senescence, and apoptosis. It was shown that RNF8, a RING-finger ubiquitin ligase, rapidly assembles at DSBs by the interaction between its forkhead-associated domain and the adaptor protein MDC1 that was phosphorylated by the DNA-damage activated protein kinase ataxia telangiectasia mutated. Subsequently RNF8 catalyzes

ubiquitylation of both H2A and H2AX and facilitates the accumulation of checkpoint mediator proteins BRCA1 and 53BP1 to the damaged chromatin partly via ubiquitylated H2AX [34–36]. The report that both histones H2A and H2AX can be the substrate of RNF8 ubiquitin ligase suggests the importance of both histone H2A and H2AX ubiquitylation in double-strand break repair [36]. Further study is needed to conclude that both histone H2A and H2AX ubiquitylation by RNF8 protects genome integrity by licensing the DSB-flanking chromatin to concentrate repair factors near the DNA lesions. The report that RNF168 interacts with ubiquitylated H2A and assembles at DSBs in an RNF8-dependent manner amplifying the local concentration of Lysine 63-linked ubiquitin by targeting H2A and H2AX to the threshold required for retention of 53BP1 and BRCA1 [37,38] suggests the importance of both histone H2A and H2AX ubiquitylation in DSBs repair. It is also suggested that the interaction between RAP80 and ubiquitylated histones H2A and H2B regulated by RNF8 is increased following DNA damage and subsequently BRCA1 is translocated to DNA damage sites [39].

Whether ubiquitylation of histone H2A by RNF8 and RNF168 is involved not only in DNA double-strand break repair but also in transcriptional regulation needs to be solved in the future.

Histone H2A deubiquitylase

Monoubiquitylated histone H2A was found to be a protein whose ubiquitylation changes during hepatocyte regeneration. The liver retains the capacity to regenerate in response to changes in mass or function in both humans and animals. Following a two thirds hepatectomy, normally quiescent hepatocytes undergo one or two rounds of replication to restore the liver mass by a process of compensatory hyperplasia. A large number of genes comprise a regulatory network and are involved in liver regeneration [40–43]. The amount of monoubiquitylated core histone H2A changes dramatically during hepatocyte regeneration. Based on the concept that histone H2A deubiquitylase should be activated during hepatocyte regeneration, our group found that two ubiquitin specific proteases, designated as USP21 and USP4, increased after partial hepatectomy. Subsequently, we found that USP21 but not USP4 catalyzed the hydrolysis of mouse liver chromatin uH2A. USP21 catalyzed the hydrolysis of uH2A in nucleosome form but not that of uH2A in free form [14]. USP21 is found to activate transcription initiation using *in vitro* reconstituted chromatin [14,44,45].

In addition to USP21, Wang's group found that Ubp-M (also designated as USP16) is one of the major deubiquitylases for histone H2A. Ubp-M deubiquitylates uH2A but not uH2B *in vivo* or *in vitro*. Inhibition of Ubp-M results in decreased cell growth because of M-phase defects during the cell cycle. It has also been demonstrated that Ubp-M regulates Hox gene expression by H2A deubiquitylation and Ubp-M is involved in posterior development of Xenopus laevis. Thus, Ubp-M is one of the histone H2A deubiquitylases and is involved in cell-cycle progression and gene expression [17].

2A-DUB was identified as histone H2A deubiquitylase that activates transcription using a reporter gene as a positive co-regulator of androgen receptor (AR) [46]. 2A-DUB was shown to regulate transcription by coordinated histone acetylation and deubiquitylation. 2A-DUB interacts with p/CAF in a coregulatory protein complex participating in transcriptional regulation events in androgen receptor-dependent gene activation [46].

On the other hand, USP22 was found to be the mammalian homolog of yeast Ubp8 that was previously characterized as a subunit of the yeast SAGA transcriptional cofactor complex. Ubp8-mediated deubiquitylation of H2B resulted in altered levels of gene-associated H3 Lys 4 methylation and Lys 36 methylation, which have both been linked to transcription [21,47,48]. It is known that H2B is the only histone ubiquitylated in S. cerevisiae [18] and the yeast SAGA transcriptional cofactor complex hydrolyzes ubiquitylated H2B. In contrast to the yeast SAGA complex, the TFTC/STAGA metazoan complex containing GCN5 HAT was found to contain deubiquitylase activity of uH2A in addition to that of uH2B. The deubiquitylase activity of the TFTC/STAGA HAT complex is necessary to counteract heterochromatin silencing and acts as a positive cofactor for activation by nuclear receptors *in vivo* [48]. Furthermore, USP22 is recruited to specific genes by activators such as the Myc oncoprotein essential for cell growth. Thus, USP22 is required for progression of the cell cycle [47].

In addition to deubiquitylases such as USP21, Ubp-M, USP22 and 2A-DUB acting as transcriptional regulators, USP3 was found to be a deubiquitylase of uH2A to maintain genome stability [49,50]. It was found that USP3 acts as a deubiquitylating enzyme for uH2A and uH2B. USP3 dynamically associates with chromatin and deubiquitylates uH2A/uH2B *in vivo*. The ZnF-UBP domain of USP3 mediates uH2A–USP3 interaction and USP3 inhibition leads to a delay in S phase progression and to accumulation of DNA breaks. It was shown that in response to ionizing radiation, changed ubiquitylation and deubiquitylation of H2A are observed and ubiquitylated H2A colocalizes in gamma-H2AX DNA repair foci. It was also shown that USP3 is required for full deubiquitylation of ubiquitin-conjugates including uH2A and gamma-H2AX dephosphorylation. Thus, it has been suggested that USP3 plays a novel role in the maintenance of genome integrity [49].

Since various H2A ubiquitin ligases/deubiquitylases have been identified in specific biological phenomena, it can be suggested that H2A ubiquitylation represents a widely used mechanism for transcriptional regulation and tuning of genome stability.

Histone H2A ubiquitylation and transcriptional regulation

Regarding histone H2A ubiquitylation and biological phenomena, several reports described a relationship between diverse phenomena and ubiquitylation of H2A. There was initially reported to be an average of 0.4–1.0 molecules of ubiquitylated H2A (uH2A) per 10 nucleosomes of dividing myoblasts and myotubes but to be reduced in resting (G0) and differentiated cells [51]. It was also reported that both uH2A and uH2B disappear during the G2 to M-phase transition when chromatin becomes condensed, but reappear as chromatin decondenses at the M to G1 transition [52] Using a monoclonal antibody recognizing uH2A, it was found that non-proliferating cells displayed lower levels of uH2A than their proliferating counterparts. Transformed cells contained clusters of uH2A that were quite abundant and showed variable size, shape and distribution even within a single cell line. The clusters did not colocalize with other known nuclear antigens and were rare in normal cells. In addition, uH2A partly co-localized with PCNA replication foci, suggesting a role for uH2A in DNA replication [53]. However, ubiquitylated

H2A were originally found in quiescent liver cells using isotope labeling of a protein that decreases during liver cell regeneration, corresponding to DNA replication [10,54]. Recently, it has been confirmed that quiescent liver cells contain more ubiquitylated H2A compared to regenerating dividing hepatocytes using antibodies [14]. Dynamic changes in uH2A were observed in a liver regeneration model and in primary prostate tumors compared to normal tissues [14,46]. It is also reported that ubiquitylated H2A is present throughout the cell cycle in proliferating cells, but ubiquitylation was reduced during G2/M transition and is hardly detectable on condensed chromosomes [17,55]. Thus, complexity of the link between uH2A and cell growth needs to be clarified in the future.

Because of the relationship between ubiquitylation of H2A and diverse phenomena such as replication, recombination and transcription, ubiquitylated H2A plays multiple roles in metazoan cells. Recently, several reported investigations have clarified that one of the major roles ascribed to ubiquitylated H2A is transcriptional regulation. A report that purified human Polycomb repressive complex 1-like protein (hPRC1L) and its subunit Ring2/Ring1B contained E3 ubiquitin ligase activity specific for histone H2A suggests a linkage between transcriptional repression and ubiquitylation of H2A. Chromatin immunoprecipitation analysis demonstrated colocalization of dRing with ubiquitylated H2A at the PRE and promoter regions of the Drosophila Ubx gene in wing imaginal discs. Knockout of dRing using tissue culture cells by RNA interference resulted in the loss of H2A ubiquitylation concomitant with derepression of Ubx. Thus, these studies revealed a linkage between H2A ubiquitylation and Polycomb silencing [32]. Using Ubp-M knockdown in HeLa cells, it was shown that increased uH2A levels at the promotor of the HoxD10 gene accompanied gene repression. Wildtype but not catalytic mutant Ubp-M could rescue HoxD10 expression. The function of Ubp-M was shown to be essential for posterior development in X. laevis [17]. Using the inactive X chromosome in female mammals it was shown that uH2A that occurred on the inactive X chromosome correlates with recruitment of Polycomb repressor complex 1 (PRC1). On the inactive X chromosome, uH2A was maintained in Ring1A or Ring1B null cells, but not in double knockout cells, demonstrating an overlapping function for these proteins in development. These observations link H2A ubiquitylation, X inactivation and PRC1 function, suggesting an unanticipated and novel mechanism for chromatin-mediated heritable gene silencing. The finding that H2A ubiquitylation is required for PcG protein-regulated gene regulation suggests that it may be part of the mechanism underlying PcG protein-regulated gene silencing [56].

Using regenerating liver used in the discovery of ubiquitylated H2A, the significance of ubiquitylated H2A was clarified in chromatin. It was found that there is a strong correlation between the levels of uH2A and trimethyl H3K9 known to be associated with transcriptional repression. These findings support a role for uH2A in silent chromatin *in vivo*.

To analyze the significance of ubiquitylation of H2A *in vitro*, H3–H4 tetramers and H2A–H2B and uH2A–H2B dimers were purified from mouse liver nuclei and the chromatin was assembled using NAP-1 and ACF [44]. There were no differences in the nucleosome repeat length or the efficiency of assembly between chromatin with either H2A–H2B dimers and ubiquitylated H2A–H2B dimers. However, *in vitro* experiments showed the presence of a trans-histone crosstalk that ubiquitylation of H2A inhibits di- and tri-methylation of H3K4 by MLL3. These studies showed the mechanisms of how uH2A represses transcription.

In addition it was shown that uH2A is engaged in the initiation step of transcription by *in vitro* studies [14]. Transcriptional activation was assayed on reconstituted chromatin templates using crude nuclear extracts as a source of RNA polymerase II. Ubiquitylation of H2A inhibited transcription if the chromatin template was assembled before preinitiation complex formation composed of general transcription factors and the RNA polymerase II occurred. On this reconstituted chromatin, ubiquitylated H2A prevented H3K4 di- and tri-methylation by methyltransferase, and USP21 could relieve this inhibition by deubiquitylating uH2A (Fig. 1). Interestingly, if chromatin is assembled with mutant histone H3 Arginine 4 instead of Lysine 4, initiation and elongation were allowed even in the presence of uH2A. Since under these conditions transcriptional initiation and elongation occurred normally, it indicated that ubiquitylation of H2A does inhibit preinitiation complex formation, not in a direct way but indirectly by preventing H3K4 methylation. These observations demonstrated the mechanisms of how ubiquitylation of H2A inhibit transcriptional initiation but not elongation *in vitro*.

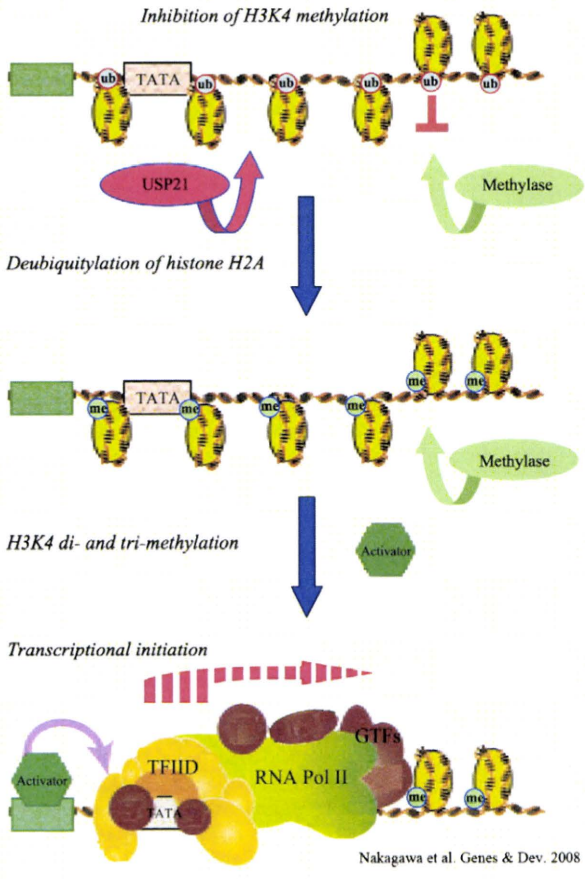

Fig. 1 – Model of transcriptional initiation from chromatin template. H3K4 methylation is essential for preinitiation complex formation. Ubiquitylation of H2A does inhibit preinitiation complex formation, not in a direct way but indirectly by preventing H3K4 methylation. Deubiquitylation of histone H2A by USP21 activates transcriptional initiation via trans-histone crosstalk with H3K4 di- and tri-methylation.

Perspective

Diverse biological phenomena are controlled by histone H2A ubiquitylation and histone crosstalk. In addition, these trans-histone crosstalks may depend on a specific sequence of interactions, and multi H2A ubiquitylation and deubiquitylation machineries may act to generate diversity in biological phenomena such as transcription. These points need to be investigated further, considering the different cis- and trans-elements and the multiple trans-histone crosstalk networks.

REFERENCES

[1] S.L. Berger, Histone modifications in transcriptional regulation, Curr. Opin. Genet. Dev. 12 (2002) 142–148.
[2] J. Mellor, The dynamics of chromatin remodeling at promoters, Mol. Cell 19 (2005) 147–157.
[3] T. Kouzarides, Chromatin modifications and their function, Cell 128 (2007) 693–705.
[4] G. Orphanides, D. Reinberg, A unified theory of gene expression, Cell 108 (2002) 439–451.
[5] J.T. Kadonaga, Eukaryotic transcription: an interlaced network of transcription factors and chromatin-modifying machines, Cell 92 (1998) 307–313.
[6] K. Luger, A.W. Mader, R.K. Richmond, D.F. Sargent, T.J. Richmond, Crystal structure of the nucleosome core particle at 2.8 A resolution, Nature 389 (1997) 251–260.
[7] W. Fischle, Y. Wang, C.D. Allis, Histone and chromatin cross-talk, Curr. Opin. Cell Biol. 15 (2003) 172–183.
[8] B.D. Strahl, C.D. Allis, The language of covalent histone modifications, Nature 403 (2000) 41–45.
[9] A. Hershko, A. Ciechanover, The ubiquitin system, Annu. Rev. Biochem. 67 (1998) 425–479.
[10] I.L. Goldknopf, C.W. Taylor, R.M. Baum, L.C. Yeoman, M.O. Olson, A.W. Prestayko, H. Busch, Isolation and characterization of protein A24, a "histone-like" non-histone chromosomal protein, J. Biol. Chem. 250 (1975) 7182–7187.
[11] I.L. Goldknopf, H. Busch, Isopeptide linkage between nonhistone and histone 2A polypeptides of chromosomal conjugate-protein A24, Proc. Natl. Acad. Sci. U. S. A. 74 (1977) 864–868.
[12] J. Barsoum, A. Varshavsky, Preferential localization of variant nucleosomes near the 5′-end of the mouse dihydrofolate reductase gene, J. Biol. Chem. 260 (1985) 7688–7697.
[13] L. Levinger, A. Varshavsky, Selective arrangement of ubiquitinated and D1 protein-containing nucleosomes within the Drosophila genome, Cell 28 (1982) 375–385.
[14] T. Nakagawa, T. Kajitani, S. Togo, N. Masuko, H. Ohdan, Y. Hishikawa, T. Koji, T. Matsuyama, T. Ikura, M. Muramatsu, T. Ito, Deubiquitylation of histone H2A activates transcriptional initiation via trans-histone cross-talk with H3K4 di- and trimethylation, Genes Dev. 22 (2008) 37–49.
[15] Y. Zhang, Transcriptional regulation by histone ubiquitination and deubiquitination, Genes Dev. 17 (2003) 2733–2740.
[16] A. Shilatifard, Chromatin modifications by methylation and ubiquitination: implications in the regulation of gene expression, Annu. Rev. Biochem. 75 (2006) 243–269.
[17] H. Joo, L. Zhai, C. Yang, S. Nie, H. Erdjument-Bromage, P. Tempst, C. Chang, H. Wang, Regulation of cell cycle progression and gene expression by H2A deubiquitination, Nature 449 (2007) 1068–1072.
[18] K. Robzyk, J. Recht, M.A. Osley, Rad6-dependent ubiquitination of histone H2B in yeast, Science 287 (2000) 501–504.
[19] M.H. West, W.M. Bonner, Histone 2B can be modified by the attachment of ubiquitin, Nucleic Acids Res. 8 (1980) 4671–4680.
[20] A.W. Thorne, P. Sautiere, G. Briand, C. Crane-Robinson, The structure of ubiquitinated histone H2B, EMBO J. 6 (1987) 1005–1010.
[21] K.W. Henry, A. Wyce, W.S. Lo, L.J. Duggan, N.C. Emre, C.F. Kao, L. Pillus, A. Shilatifard, M.A. Osley, S.L. Berger, Transcriptional activation via sequential histone H2B ubiquitylation and deubiquitylation, mediated by SAGA-associated Ubp8, Genes Dev. 17 (2003) 2648–2663.
[22] C.F. Kao, C. Hillyer, T. Tsukuda, K. Henry, S. Berger, M.A. Osley, Rad6 plays a role in transcriptional activation through ubiquitylation of histone H2B, Genes Dev. 18 (2004) 184–195.
[23] T. Xiao, C.F. Kao, N.J. Krogan, Z.W. Sun, J.F. Greenblatt, M.A. Osley, B.D. Strahl, Histone H2B ubiquitylation is associated with elongating RNA polymerase II, Mol. Cell. Biol. 25 (2005) 637–651.
[24] L. Hicke, Protein regulation by monoubiquitin, Nat. Rev. Mol. Cell Biol. 2 (2001) 195–201.
[25] L. Hicke, R. Dunn, Regulation of membrane protein transport by ubiquitin and ubiquitin-binding proteins, Annu. Rev. Cell Dev. Biol. 19 (2003) 141–172.
[26] C.M. Pickart, M.J. Eddins, Ubiquitin: structures, functions, mechanisms, Biochim. Biophys. Acta 1695 (2004) 55–72.
[27] S. Matsui, B. Seon, A. Sandberg, Disappearance of a structural chromatin protein A24 in mitosis: implications for molecular basis of chromatin condensation, Proc. Natl. Acad. Sci. U. S. A. 76 (1979) 6386–6390.
[28] B. Pina, P. Suau, Core histone variants and ubiquitinated histones 2A and 2B of rat cerebral cortex neurons, Biochem. Biophys. Res. Commun. 133 (1985) 505–510.
[29] L. Bohm, C. Crane-Robinson, P. Sautiere, Proteolytic digestion studies of chromatin core-histone structure. Identification of a limit peptide of histone H2A, Eur. J. Biochem. 106 (1980) 525–530.
[30] L.J. Jason, R.M. Finn, G. Lindsey, J. Ausio, Histone H2A ubiquitination does not preclude histone H1 binding, but it facilitates its association with the nucleosome, J. Biol. Chem. 280 (2005) 4975–4982.
[31] P.S. Swerdlow, T. Schuster, D. Finley, A conserved sequence in histone H2A which is a ubiquitination site in higher eucaryotes is not required for growth in Saccharomyces cerevisiae, Mol. Cell. Biol. 10 (1990) 4905–4911.
[32] H. Wang, L. Wang, H. Erdjument-Bromage, M. Vidal, P. Tempst, R.S. Jones, Y. Zhang, Role of histone H2A ubiquitination in Polycomb silencing, Nature 431 (2004) 873–878.
[33] W. Zhou, P. Zhu, J. Wang, G. Pascual, K.A. Ohgi, J. Lozach, C.K. Glass, M.G. Rosenfeld, Histone H2A monoubiquitination represses transcription by inhibiting RNA polymerase II transcriptional elongation, Mol. Cell 29 (2008) 69–80.
[34] M.S. Huen, R. Grant, I. Manke, K. Minn, X. Yu, M.B. Yaffe, J. Chen, RNF8 transduces the DNA-damage signal via histone ubiquitylation and checkpoint protein assembly, Cell 131 (2007) 901–914.
[35] N.K. Kolas, J.R. Chapman, S. Nakada, J. Ylanko, R. Chahwan, F.D. Sweeney, S. Panier, M. Mendez, J. Wildenhain, T.M. Thomson, L. Pelletier, S.P. Jackson, D. Durocher, Orchestration of the DNA-damage response by the RNF8 ubiquitin ligase, Science 318 (2007) 1637–1640.
[36] N. Mailand, S. Bekker-Jensen, H. Faustrup, F. Melander, J. Bartek, C. Lukas, J. Lukas, RNF8 ubiquitylates histones at DNA double-strand breaks and promotes assembly of repair proteins, Cell 131 (2007) 887–900.
[37] C. Doil, N. Mailand, S. Bekker-Jensen, P. Menard, D.H. Larsen, R. Pepperkok, J. Ellenberg, S. Panier, D. Durocher, J. Bartek, J. Lukas, C. Lukas, RNF168 binds and amplifies ubiquitin conjugates on damaged chromosomes to allow accumulation of repair proteins, Cell 136 (2009) 435–446.
[38] S. Pinato, C. Scandiuzzi, N. Arnaudo, E. Citterio, G. Gaudino, L. Penengo, RNF168, a new RING finger, MIU-containing protein that modifies chromatin by ubiquitination of histones H2A and H2AX, BMC Mol. Biol. 10 (2009) 55.

[39] J. Wu, M.S. Huen, L.Y. Lu, L. Ye, Y. Dou, M. Ljungman, J. Chen, X. Yu, Histone ubiquitination associates with BRCA1-dependent DNA damage response, Mol. Cell. Biol. 29 (2009) 849–860.
[40] R.H. Costa, V.V. Kalinichenko, A.X. Holterman, X. Wang, Transcription factors in liver development, differentiation, and regeneration, Hepatology 38 (2003) 1331–1347.
[41] N. Fausto, J.S. Campbell, K.J. Riehle, Liver regeneration, Hepatology 43 (2006) S45–53.
[42] D. Mangnall, N.C. Bird, A.W. Majeed, The molecular physiology of liver regeneration following partial hepatectomy, Liver Int. 23 (2003) 124–138.
[43] G.K. Michalopoulos, M.C. DeFrances, Liver regeneration, Science 276 (1997) 60–66.
[44] T. Ito, M. Bulger, M.J. Pazin, R. Kobayashi, J.T. Kadonaga, ACF, an ISWI-containing and ATP-utilizing chromatin assembly and remodeling factor, Cell 90 (1997) 145–155.
[45] T. Ito, M.E. Levenstein, D.V. Fyodorov, A.K. Kutach, R. Kobayashi, J.T. Kadonaga, ACF consists of two subunits, Acf1 and ISWI, that function cooperatively in the ATP-dependent catalysis of chromatin assembly, Genes Dev. 13 (1999) 1529–1539.
[46] P. Zhu, W. Zhou, J. Wang, J. Puc, K. Ohgi, H. Erdjument-Bromage, P. Tempst, C. Glass, M. Rosenfeld, A histone H2A deubiquitinase complex coordinating histone acetylation and H1 dissociation in transcriptional regulation, Mol. Cell 27 (2007) 609–621.
[47] X.Y. Zhang, M. Varthi, S.M. Sykes, C. Phillips, C. Warzecha, W. Zhu, A. Wyce, A.W. Thorne, S.L. Berger, S.B. McMahon, The putative cancer stem cell marker USP22 is a subunit of the human SAGA complex required for activated transcription and cell-cycle progression, Mol. Cell 29 (2008) 102–111.
[48] Y. Zhao, G. Lang, S. Ito, J. Bonnet, E. Metzger, S. Sawatsubashi, E. Suzuki, X. Le Guezennec, H.G. Stunnenberg, A. Krasnov, S.G. Georgieva, R. Schule, K. Takeyama, S. Kato, L. Tora, D. Devys, A TFTC/STAGA module mediates histone H2A and H2B deubiquitination, coactivates nuclear receptors, and counteracts heterochromatin silencing, Mol. Cell 29 (2008) 92–101.
[49] F. Nicassio, N. Corrado, J.H. Vissers, L.B. Areces, S. Bergink, J.A. Marteijn, B. Geverts, A.B. Houtsmuller, W. Vermeulen, P.P. Di Fiore, E. Citterio, Human USP3 is a chromatin modifier required for S phase progression and genome stability, Curr. Biol. 17 (2007) 1972–1977.
[50] K.E. Sloper-Mould, H.J. Eyre, X.W. Wang, G.R. Sutherland, R.T. Baker, Characterization and chromosomal localization of USP3, a novel human ubiquitin-specific protease, J. Biol. Chem. 274 (1999) 26878–26884.
[51] A. Wunsch, A. Haas, J. Lough, Synthesis and ubiquitination of histones during myogenesis, Dev. Biol. 119 (1987) 85–93.
[52] R.S. Wu, K.W. Kohn, W.M. Bonner, Metabolism of ubiquitinated histones, J. Biol. Chem. 256 (1981) 5916–5920.
[53] A. Vassilev, H. Rasmussen, E. Christensen, S. Nielsen, J. Celis, The levels of ubiquitinated histone H2A are highly upregulated in transformed human cells: partial colocalization of uH2A clusters and PCNA/cyclin foci in a fraction of cells in S-phase, J. Cell Sci. 108 (1995) 1205–1215.
[54] M.O. Olson, I.L. Goldknopf, K.A. Guetzow, G.T. James, T.C. Hawkins, C.J. Mays-Rothberg, H. Busch, The NH2- and COOH-terminal amino acid sequence of nuclear protein A24, J. Biol. Chem. 251 (1976) 5901–5903.
[55] R. Mueller, H. Yasuda, C. Hatch, W. Bonner, E. Bradbury, Identification of ubiquitinated histones 2A and 2B in Physarum polycephalum. Disappearance of these proteins at metaphase and reappearance at anaphase, J. Biol. Chem. 260 (1985) 5147–5153.
[56] M. de Napoles, J.E. Mermoud, R. Wakao, Y.A. Tang, M. Endoh, R. Appanah, T.B. Nesterova, J. Silva, A.P. Otte, M. Vidal, H. Koseki, N. Brockdorff, Polycomb group proteins Ring1A/B link ubiquitylation of histone H2A to heritable gene silencing and X inactivation, Dev. Cell 7 (2004) 663–676.

A histone chaperone, DEK, transcriptionally coactivates a nuclear receptor

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Chromatin reorganization is essential for transcriptional control by sequence-specific transcription factors. However, the molecular link between transcriptional control and chromatin reconfiguration remains unclear. By colocalization of the nuclear ecdysone receptor (EcR) on the ecdysone-induced puff in the salivary gland, *Drosophila* DEK (dDEK) was genetically identified as a coactivator of EcR in both insect cells and intact flies. Biochemical purification and characterization of the complexes containing fly and human DEKs revealed that DEKs serve as histone chaperones via phosphorylation by forming complexes with casein kinase 2. Consistent with the preferential association of the DEK complex with histones enriched in active epigenetic marks, dDEK facilitated H3.3 assembly during puff formation. In some human myeloid leukemia patients, DEK was fused to CAN by chromosomal translocation. This mutation significantly reduced formation of the DEK complex, which is required for histone chaperone activity. Thus, the present study suggests that at least one histone chaperone can be categorized as a type of transcriptional coactivator for nuclear receptors.

[*Keywords*: DEK; acute myeloid leukemia; histone chaperone; ecdysone receptor; coactivator; histone variant H3.3]

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Chromatin structure is reorganized during gene activation through chromatin remodeling and epigenetic modification (Henikoff 2008; Jiang and Pugh 2009). Through these processes, nucleosomal DNA becomes accessible to sequence-specific transcription factors, facilitating their stable binding at specific sites in target gene promoters (Cairns 2009). Chromatin reconfiguration supporting transcriptional initiation and subsequent pre-mRNA elongation requires a number of functionally distinct regulatory complexes (Narlikar et al. 2002; Roeder 2005; Rosenfeld et al. 2006). One such class is an ATP-dependent chromatin remodeling complex. This class of complexes uses ATP hydrolysis to directly rearrange nucleosomal arrays in a noncovalent manner by sliding and transferring histone octamers associating with chromosomal DNA (Kouzarides 2007; Morrison and Shen 2009). Another class of regulatory complexes can be categorized as histone-modifying enzymes, which epigenetically modify histone proteins (Borrelli et al. 2008; Sims and Reinberg 2008). These enzymes covalently supply reversible epigenetic marks (acetyl groups, methyl groups, and phosphates) at specific residues on histone tails. Combinations of histone modifications generate a "histone code" to direct chromatin configuration in surrounding chromatin areas (Strahl and Allis 2000; Kouzarides 2007). Moreover, the eviction and reassembly of histone octamers at transcribing gene loci are evident during chromatin reconfiguration, and require a third class of nucleosomal regulators: histone chaperones (De Koning et al. 2007; Park and Luger 2008). In this regard, histone chaperones are believed to transcriptionally coregulate the function of sequence-specific regulators because core histones are mobilized during the transcription and elongation process (Adkins et al. 2004; Adkins and Tyler 2006). Specific deposition of histone variants like histone H3.3 is also well documented in transcriptionally active loci in *Drosophila* cells (Mito et al. 2005; Schwartz and Ahmad 2005; Henikoff 2008), but the histone chaperones involved in this deposition have not been identified. The exact form of histone chaperone units and their mode of function appear diverse. However,

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the role of each histone chaperone in the processes of transcriptional control by sequence-specific regulators is poorly understood.

An insect steroid hormone, ecdysone, induces metamorphosis (Thummel 1996). Like mammalian nuclear receptors (NRs) (Evans 1988; Green and Chambon 1988), nuclear ecdysone receptor (EcR) has been characterized as a ligand-dependent and sequence-specific transcriptional activator, and heterodimerizes with ultraspiracle (USP) to control target gene expression in an ecdysone-dependent manner (Koelle et al. 1991; King-Jones and Thummel 2005). Not surprisingly, key transcriptional coregulators are functionally and structurally conserved from insects to mammals (Bai et al. 2000; Takeyama et al. 2002; Sedkov et al. 2003). This observation suggests that chromatin reconfiguration might be essential for EcR-mediated transcriptional control, as observed previously for mammalian NRs. In this respect, ecdysone-induced puff formation in the salivary gland of the fly (Ashburner 1990; Thummel 2002) is a readily observed example of chromatin reorganization induced by NRs. Although the prominent morphological alteration of chromatin structure was initially described decades ago (Ashburner 1967), the molecular basis and the associated regulatory factors are scarcely known. In the present study, we used *Drosophila* genetic screening to identify regulators supporting chromatin reorganization induced by liganded EcR. We found that a chaperone, *Drosophila* DEK (dDEK), is colocalized with EcR at the ecdysone-induced puff, and acts as a transcriptional EcR coactivator. Biochemical purification and characterization of fly and human DEK (hDEK) complexes revealed that phosphorylated DEKs associating with casein kinase 2 (CK2) serve as a histone chaperone. Moreover, in a group of acute myeloid leukemia (AML) patients, a mutant hDEK protein is known to be fused with CAN (Soekarman et al. 1992; von Lindern et al. 1992). We found the mutant to be defective in chaperone activity. Thus, the present study suggests that a specific class of histone chaperones serves as a NR coactivator.

Results

Genetic screening identified dDEK as an ecdysone-inducible puff-localized factor

To identify a regulator involved in ecdysone-induced puff formation in the salivary gland of *Drosophila*, we genetically screened candidates from EGFP protein trap library lines (Morin et al. 2001; Buszczak et al. 2007) treated with a synthetic ecdysone: Muristerone A (Mur) (Supplemental Fig. S1A). In the induced puff, several candidate lines colocalizing EGFP with EcR were identified. Among them, two lines—G00131 (Fig. 1A) and CA06616 (Supplemental Fig. S1B; Morin et al. 2001; Buszczak et al. 2007)—were selected for further analysis. The sequence of the flanking genomic DNA and the transcripts from these two lines led us to identify the same gene (Fig. 1B), the *Drosophila* ortholog (*dDEK*) of the *hDEK* oncogene (von Lindern et al. 1992). To characterize endogenous dDEK expression in the salivary gland, we generated a polyclonal antibody against dDEK (Supplemental Fig. S1C). Staining of polytene chromosomes from wild-type larvae with the antibody showed that dDEK and EcR overlapped on puffs (Supplemental Fig. S2A). Based on the immunofluorescence of polytene chromosomes with anti-Ser5-phosphorylated RNA polymerase II (Pol II) (Weeks et al. 1993), dDEK appeared to be associated with transcriptionally active loci (Fig. 1C). dDEK was seen in the less-compact chromatin interbands (estimated as weak DAPI staining), and its location was the converse of that of histone H1, a marker of condensed chromatin (Fig. 1D; Supplemental Fig. S2B; Kim et al. 2004). These findings suggested that dDEK was localized in regions of transcriptionally active chromatin.

dDEK is an EcR coactivator

We examined the possible association of dDEK with EcR in *Drosophila* S2 cells. dDEK was coimmunoprecipitated with EcR in the absence or presence of Mur (Fig. 2A). Next, physical interaction was tested by a pull-down assay using S-tagged dDEK with EcR recombinant protein prepared in a baculoviral expression system. Ligand-independent association of EcR with dDEK was seen even at high NaCl concentration (Fig. 2B). Thus, dDEK appeared to be a ligand-independent interactant for EcR.

To determine if dDEK was functionally involved in ecdysone-induced chromatin reorganization, we tested the impact of dDEK in ecdysone-induced gene expression in intact flies. We used an RNAi approach with the GAL4-UAS binary system to knock down endogenous dDEK (Pili-Floury et al. 2004). We established UAS inverted repeat (IR) transgenic strains, carrying fragments of the *dDEK* gene to form dsRNA to target the dDEK transcript under control of a salivary gland-specific GAL4 driver (*sgs3-GAL4*) (Stabell et al. 2007). Likewise, either EcR or Taiman (Tai) was knocked down in the salivary glands (Supplemental Fig. S3A). As anticipated, in vitro treatment of wild-type flies' salivary glands with Mur induced expression of EcR target genes (*Eip74EF*, *Eip75B*, and *BR-C*) located within puff regions (Fig. 2C; Supplemental Fig. S4A; Burtis et al. 1990; Karim and Thummel 1992). However, knockdown of either dDEK (IR-dDEK) or EcR (IR-EcR) resulted in the loss of the Mur response, which was also observed following knockdown of Tai (IR-Tai) (Fig. 2C; Supplemental Fig. S4A). Taiman, a *Drosophila* homolog of human *AIB1* histone acetyltransferase (HAT), is known to coactivate EcR (Bai et al. 2000). Thus, it appeared that dDEK was a transcriptional EcR coactivator. Using the same knockdown approach with Mur-treated S2 cells (Supplemental Fig. S3B), we verified a significant role of dDEK in EcR-mediated gene induction (Supplemental Fig. S4B).

To directly test if dDEK coactivated the transactivation function of EcR, the coregulator role of dDEK was tested in a transient luciferase reporter assay (Sawatsubashi et al. 2004). Overexpression of dDEK coactivated EcR in the presence of Mur (Fig. 2D, lanes 3,4), without potentiation of the basal promoter activity (Fig. 2D, lanes 1,2), consistent

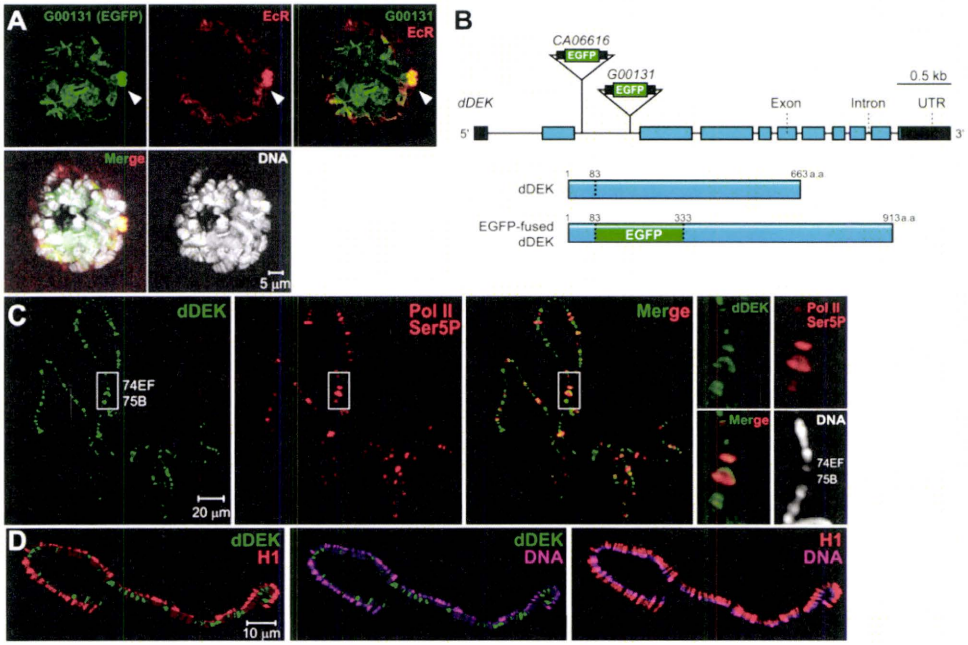

Figure 1. Localization of dDEK within the ecdysone-induced puff. (*A*) Third instar larval salivary gland nuclei of the G00131 protein trap line in which the EGFP-fused protein (green) colocalizes with the EcR (marked by an anti-EcR antibody, stained red) at ecdysone-induced puff loci (arrowhead) and interbands on polytene chromosomes (DNA stain, white). (*B*) Insertion sites of protein trap lines in G00131 and CA06616. (*Top*) The dDEK transcript fused to the EGFP exon (green box) carried by line G00131 or CA06616. The untranslated region (black), coding regions (blue), and insertion alleles (triangle) are shown. (*Bottom*) Schematic representation of deduced EGFP-fused dDEK proteins expressed in G00131 is also shown. (*C*) Immunolocalization of dDEK on wild-type polytene chromosomes. The wild-type polytene chromosome squashes are costained with anti-dDEK (green) and anti-RNA polymerase II Ser5-P (as a maker of puff loci) antibodies (red) and DAPI (DNA stain, white). Endogenous dDEK is enriched at specific polytene chromosome sites, including ecdysone-induced puffs at 74EF and 75B (white box). The *right* panels present higher-magnification images of the white-boxed areas. (*D*) The distributions of dDEK (green) and H1 (as a marker of condensed chromatin, red) are shown in split images, including costaining for DNA (magenta). dDEK primarily associated with interband regions.

with knockdown assays using dsRNA (Fig. 2D, lanes 5,7). Similarly, transactivation was attenuated when the known EcR coactivator Tai was knocked down (Fig. 2D, lane 6).

To determine if the observed association of EcR with dDEK also occurred at the EcR target gene promoter, the endogenous promoter of the *Eip75B* gene in S2 cells was subjected to chromatin immunoprecipitation (ChIP) analysis. Although the EcR-binding sites remain to be mapped in this promoter, an EcR-binding site was found in the first intron region designated as position E, after testing regions A–J (see Supplemental Fig. S4C). EcR was recruited upon Mur treatment, and recruitment of dDEK was also inducible by Mur at site E (Fig. 2E). When EcR was knocked down in S2 cells, recruitment of dDEK was abolished. In contrast, dDEK was not indispensable for ligand-induced EcR recruitment (Fig. 2E).

Biochemical identification of a dDEK complex

Regulators reorganizing chromatin's configuration often form nuclear multisubunit complexes. Thus, a dDEK-containing complex was biochemically purified from the nuclear extracts (NEs) of a newly established stable S2 transformant expressing Flag-tagged dDEK (e-dDEK). A dDEK-containing complex was purified through several chromatographic steps (Fig. 3A), and the components were analyzed using MALDI-TOF/mass spectrometry (MS) (Ohtake et al. 2007; Fujiki et al. 2009). The α and β subunits of *Drosophila* CK2 (dCK2) were copurified and identified together with dDEK (Fig. 3B, top panel), and were further verified by Western blot (Fig. 3B, bottom panel). The purified dDEK–CK2 complex [dDEK (NE) com] exhibited a size of ~670 kDa by gel filtration, and dDEK was phosphorylated (Fig. 3C, bottom panel). Consistent with the isolation of the dDEK–CK2 complex, colocalization of dCK2α with dDEK and EcR was detected in the puff regions on polytene chromosome (Supplemental Fig. S5A,B).

Functional kinase activity of CK2 is achieved through formation of a heterotetramer containing the catalytic α subunit and the regulatory β subunit (~130 kDa) (Litchfield 2003). As bacterially expressed dDEK recombinant proteins

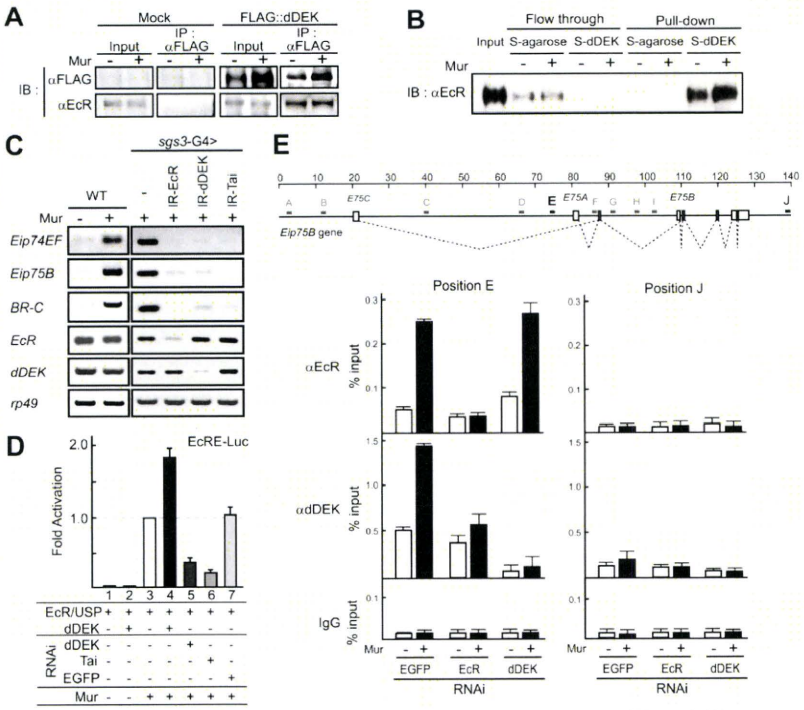

Figure 2. dDEK acts as a coactivator of the EcR. (*A*) Association of dDEK with EcR in S2 cells. NEs prepared from either S2 cells (Mock) or S2 cells stably expressing Flag-tagged dDEK (e-dDEK) were subjected to immunoprecipitation (IP) with an anti-Flag antibody. EcR was detected by Western blotting using an anti-EcR antibody. (*B*) Direct interaction of dDEK with EcR in pull-down assay. EcR and USP were incubated with S-tagged dDEK immobilized on beads in the absence or presence of Mur (2.5×10^{-7} M). Flowthrough fractions and bound fractions were analyzed by Western blotting using an anti-EcR antibody. (*C*) Semiquantitative RT–PCR analysis of EcR target genes in third instar larval salivary glands untreated (−) or treated with Mur (+) for 2 h. RT–PCR was performed for *Eip74EF*, *Eip75B*, *BR-C*, *EcR*, *dDEK*, and *rp49* (as a control) in RNA isolated from salivary glands expressing IR-EcR, IR-dDEK, or IR-Tai transgene for RNAi-mediated knockdown. The knockdown efficiencies are shown in Supplemental Figure S3A. (*D*) Coactivation of liganded EcR function by dDEK in S2 cells. For reporter assays, S2 cells were transfected with indicated plasmid (EcRE-Luc, EcR, and USP with or without dDEK) and then incubated for 16 h in the absence or presence of Mur (2.5×10^{-7} M). For knockdown of endogenous dDEK or Tai (EGFP as a negative control), the cells were preliminarily transfected with dsRNA followed by incubation for 72 h. The knockdown efficiencies are shown in Supplemental Figure S3B. Results are given as means ± SD of at least three independent experiments. (*E*) Ligand-dependent recruitment of dDEK with EcR on specific sites of the *Eip75B* gene. The map of Eip75B gene region is shown in the *top* panel. For ChIP analysis, soluble chromatin prepared from S2 cells treated with or without Mur (2.5×10^{-7} M) for 1 h was immunoprecipitated with indicated antibodies. Black bars (E and J) indicate positions of the amplicons for quantitative PCR. The amplicon of position J is a negative control. In the *bottom* panel, the values are expressed as a percentage of the amount of immunoprecipitated DNA normalized to respective input DNA signals. Results are given as means ± SD of at least three independent experiments.

(full-length [FL] and 215–415) also appeared to form a tetramer by Superose 6 gel filtration analysis (Supplemental Fig. S6A), glycerol gradient sedimentation (Supplemental Fig. S6B), and Blue Native PAGE (Supplemental Fig. S6C; Wittig et al. 2006), we assumed that this complex was composed of the two tetramers.

Association of dDEK with the CK2α subunit was tested in a pull-down assay with dDEK mutants (Fig. 4A; Supplemental Fig. S7). The dDEK SAP domain (282–316 amino acids), a putative DNA-binding motif, was mapped as a CK2α-interacting domain, and this association was potentiated by the presence of ATP (Fig. 4B), suggesting phosphorylation dependency in the interaction between dDEK and CK2α. Furthermore, formation of the dDEK–CK2α and dDEK–CK2β complex was detected as shifted-up bands only in the presence of ATP (Fig. 4C).

We then asked whether CK2 phosphorylated dDEK. The dDEK protein was phosphorylated by affinity-purified dCK2α/β from S2 cells as well as human CK2α/β, and this phosphorylation was clearly blocked by a CK2 inhibitor: DMAT (Fig. 4D). The triple mutant (G303A/S304A/K305A; GSK > AAA), which is clearly defective in its interaction with CK2α, was significantly less phosphorylated by CK2 than dDEK(WT) (Fig. 4E). Thus, dDEK appeared to form a complex with CK2 through the phosphorylated dDEK.

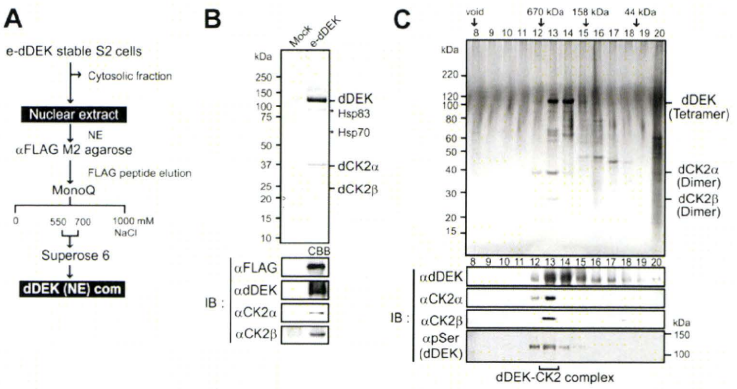

Figure 3. Identification of nuclear dDEK complex containing dCK2. (*A*) Purification scheme for the dDEK-containing complex in NEs. (*B*) Identification of the dDEK complex. (*Top* panel) Coomassie blue staining of the peak fraction of dDEK from the Mono Q purification step after anti-Flag affinity purification for subsequent MALDI-TOF/MS analysis. Subunits of the complex are identified on the *right*. Asterisks indicate background proteins. The fractions were analyzed by Western blotting using the indicated antibodies in the *bottom* panels. (*C*) Silver staining (*top*) and Western blot (*bottom*) analysis of dDEK-containing complex [dDEK (NE) com] fractionated by Superose 6 gel filtration after Mono Q purification. The elution profile of the protein markers is indicated at the *top*.

dDEK is a CK2-dependent nucleosome assembly factor

During characterization of the dDEK–CK2 complex, dDEK was found to tightly associate with chromatin, and it copurified with core histones from the soluble chromatin fraction of micrococcal nuclease (MNase)-digested nuclear pellets (Supplemental Fig. S8A,B). Immunoprecipitation of dDEK from the chromatin fraction from S2 cells revealed that phosphorylated dDEK associated with core histones (Fig. 5A, left panel), and knockdown of CK2α in S2 cells resulted in the loss of dDEK complex formation with histones (Fig. 5A, right panel). In a pull-down assay using S-tagged dDEK beads, dDEK interacted directly with native core histones purified from S2 cells, but CK2α/β was not needed for this association in vitro (Supplemental Fig. S9). However, when the histones were reconstituted with plasmid DNA, histone association with dDEK–CK2s was abolished (Supplemental Fig. S9). These findings led us to suggest that dDEK serves as a histone chaperone to assemble histones into chromatin.

To test this idea, we asked if dDEK was capable of forming a nucleosomal array. Using a reconstitution assay with native core histones purified from S2 cells and supercoiled plasmid DNA (Supplemental Fig. S10A; Ito et al. 1997), dDEK was found to transfer histones to DNA, resulting in the formation of a histone–DNA complex that was evident in the bottom fraction of the glycerol density gradient (Supplemental Fig. S10B). Furthermore, MNase digestion analysis of this bottom fraction detected formation of mono- and dinucleosomes (Supplemental Fig. S10C). Then, we asked if dDEK could replace a well-known histone chaperone: *Drosophila* NAP-1 (dNAP-1). An in vitro nucleosome assembly assay was performed with *Drosophila* ACF and dNAP-1 proteins as well as core histones (Supplemental Fig. S11) in the presence of plasmid DNA (Ito et al. 1997; Kitagawa et al. 2003). Reconstitution of the nucleosomal array by dNAP-1 histone chaperone and dACF was confirmed by MNase digestion analysis (Fig. 5B, lanes 3,4). Under these conditions, the purified dDEK–CK2 complex [dDEK (NE) com] transferred core histones to DNA (Fig. 5B, lanes 5,6). Importantly, the chaperone activity of this complex was abolished in the presence of a CK2 inhibitor: DMAT (Fig. 5B, lanes 7,8). The recombinant dDEK protein alone showed only weak nucleosome assembly activity (Fig. 5C, lanes 5,6), but, as expected, its activity was potentiated by the presence of recombinant human CK2 (hCK2) (Fig. 5C, lanes 7,8). As anticipated, the GSK > AAA mutant had no CK2-dependent histone chaperone activity (Fig. 5D, lanes 7,8). Thus, these findings suggest that dDEK requires CK2 for histone chaperone activity, achieved through formation of a functional complex via phosphorylation of dDEK.

dDEK assembles transcriptionally active chromatin

We then asked if dDEK preferentially associated with transcriptionally active or inactive histone modifications using a pull-down assay with S-tagged dDEK and the chromatin fraction from S2 cells (Supplemental Fig. S12A). Consistent with dDEK localization in transcriptionally active regions on polytene chromosomes (Fig. 1C), histones pulled down with dDEK were marked with transcriptionally active modifications such as histone H3K4 methylation (Supplemental Fig. S12B). As dDEK appeared to associate with histones in the transcriptionally active chromatin, we then asked if dDEK also interacted with the histone H3 variant H3.3. When Myc-tagged histones were overexpressed in S2 cells, dDEK was efficiently coimmunoprecipitated with H3.3, but much less with H3 (Fig. 6A). By in vitro binding assays, dDEK exhibited affinity for H2A–H2B dimers, but the association with the H3.3–H4 tetramer appeared to be more stable (Fig. 6B). To address this point under physiological conditions, H3.3's association with dDEK was tested in intact flies. dDEK's localization pattern in the salivary gland overlapped with that of H3.3, rather than that of H3 (Fig. 6C). As dDEK physically interacts with

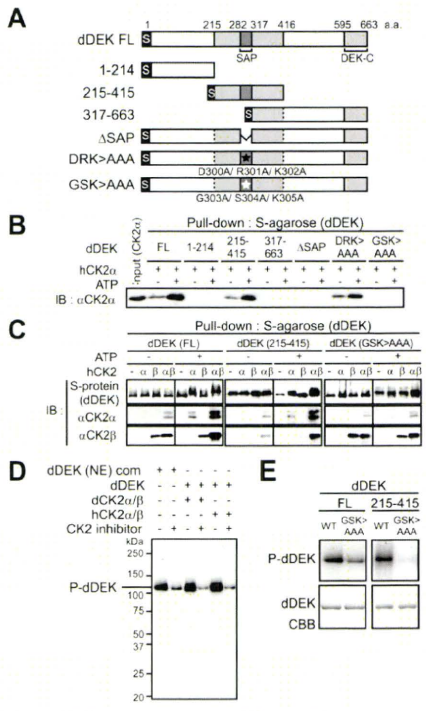

Figure 4. CK2-mediated phosphorylation of dDEK regulates formation of the dDEK–CK2 complex. (*A*) Schematic representation of dDEK domain organization of full-length (FL) and mutant variants. The gray boxes represent the evolutionarily conserved region containing SAP (pfam 02037) or DEK-C (pfam 08766) domains. For each protein, the location of N-terminal S-tag is shown. Triple-alanine mutated residues are shown as closed asterisks (D300A/R301A/K302A; DRK > AAA) or opened asterisks (G303A/S304A/K305A; GSK > AAA). (*B*) Determination of the human CK2α subunit-binding region in dDEK. For pull-down assays, recombinant hCK2α (1 μg) was incubated with S-tagged dDEK (5 μg)-immobilized beads in the absence or presence of 100 μM ATP. The pull-downs were analyzed by Western blotting using anti-CK2α antibody. (*C*) Phosphorylation-dependent formation of the dDEK–hCK2 complex. Recombinant hCK2α (1 μg) and/or hCK2β (0.6 μg) proteins were pulled down by S-tagged FL and mutant dDEK proteins with or without ATP. Bound proteins were analyzed by SDS-PAGE and Western blotting using indicated antibodies. (*D*, *E*) Phosphorylation of dDEK by CK2. In vitro kinase assays using ^{32}P-γ-ATP were performed using purified dDEK (NE) complex or indicated recombinant proteins in the absence or presence of 5 μM DMAT as CK2 inhibitor (*D*), and dDEK mutant variants (*E*). The phosphorylated products were visualized by autoradiography.

EcR, H3.3's localization was then examined in ecdysone-induced puff loci in the salivary glands (Schwartz and Ahmad 2005). As a result, H3.3 accumulation and colocalization with dDEK were observed in a Mur-dependent manner on puffs. (Fig. 6D, top panel). However, when the GSK > AAA mutant was expressed, H3.3 accumulation induced by Mur treatment was impaired, regardless of dDEK accumulation in the puff sites (Fig. 6D, bottom panel). Consistent with these observations, the Mur effect in the induction of EcR-target genes was defective in the GSK > AAA mutant (Fig. 6E; Supplemental Fig. S13). In a reporter assay, the GSK > AAA mutant lost the EcR coactivator function, and the CK2 inhibitor attenuated dDEK-mediated coactivation (Supplemental Fig. S14). Thus, it is likely that dDEK coactivated EcR through the assembly of active chromatin, enriched in transcriptionally active histone marks, within EcR target gene promoters.

The nucleosome assembly function of DEK is evolutionarily conserved between flies and humans

While the size of the hDEK protein (43 kDa) is much smaller than that of dDEK, the central and C-terminal regions of hDEK are highly conserved (Fig. 7A). Therefore, we asked if hDEK also served as a histone chaperone. First, hDEK-containing complexes were biochemically purified from a newly established stable HeLa transformant expressing Flag-His$_6$-tagged hDEK (e-hDEK) (Fig. 7B). Similar to dDEK, two types of hDEK complexes were identified. One complex contained hCK2 α/α′ and β subunits (Fig. 7C), and core histones were detected in the other fraction (Fig. 7D). By in vitro nucleosome assembly, the purified hDEK–CK2 complex was capable of forming nucleosomes (data not shown). In vitro, recombinant hDEK had significant nucleosome assembly activity in the presence of recombinant hCK2α/β, and it was attenuated by the CK2 inhibitor DMAT (Fig. 7E). Thus, like dDEK, hDEK appears to be a histone chaperone.

Impaired histone chaperone function in the leukemia DEK-CAN fusion protein

In a group of AML patients, the human DEK gene (located on chromosome 6p23) is fused to the CAN gene on chromosome 9q34 by chromosomal translocation (von Lindern et al. 1992). In such AML patients, the C-terminal domain of hDEK is spliced into the N-terminal truncated CAN (Fig. 7A), and malfunction of hDEK has been assumed. To address this issue, the histone chaperone function of hDEK-CAN was assessed. When the hDEK-CAN fusion protein was expressed in HEK 293T cells, the association of hDEK with either CK2α, CK2β, or histones was abolished (Fig. 7F). In EcR-mediated activation of the endogenous EcR target genes in intact flies, hDEK-CAN acted as a dominant-negative mutant (Fig. 7G; Supplemental Fig. S15). We attribute the disruption of functional complex formed by endogenous DEK–CK2 to hDEK/hDEK-CAN oligomerization (Fig. 7F). Thus, it appears that hDEK fusion to CAN impairs histone chaperone activity and contributes to the onset of this type of AML.

Discussion

Genetic identification of an EcR coregulator by an EGFP protein trap approach

Puff formation has long been recognized as reconfiguration of chromatin, a change that plays an important

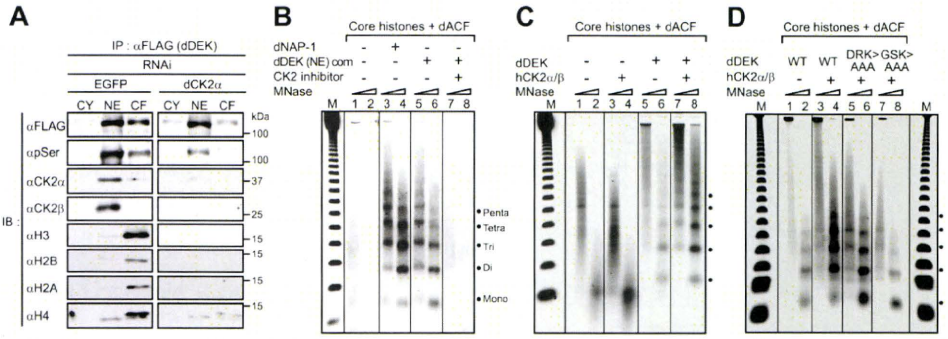

Figure 5. dDEK is a novel nucleosome assembly factor. (*A*) CK2-mediated phosphorylation of dDEK is required for association with core histones. The cytosolic fraction (CY), NE, and chromatin fraction (CF) derived from S2 cells stably expressing e-dDEK were used for immunoprecipitation using anti-Flag M2 resin. The immunoprecipitates were subjected to Western blot analysis using the indicated antibodies. For knockdown of endogenous dCK2α (*right* panel), the cells were transfected with dsRNA (EGFP as a negative control, *left* panel). The knockdown efficiencies are shown in Supplemental Figure S3C. (*B–D*) In vitro nucleosome assembly assays. (*B*) Purified dDEK–CK2 complex from NE [dDEK (NE) com] or dNAP-1 (as a positive control) were incubated with purified dACF, core histones, and plasmid DNA in the presence of CK2 inhibitor DMAT or DMSO (control) for 3 h at 27°C. The samples were partially digested with varying concentrations of MNase used to digest the chromatin. The resulting DNA fragments were visualized by ethidium bromide staining. The molecular mass markers (M) are the 123-bp ladder. The nucleosome positions are indicated at the *right*. Recombinant dDEK (*C*) and dDEK mutants (*D*) were subjected to the same assay as *B*. dDEK (DRK > AAA) protein is used as a negative control.

physiologic role at the molecular level (Ashburner 1967, 1990). However, factors responsible for the reconfiguration have remained largely unidentified. The steroid hormone ecdysone induces puff formation, and we genetically screened for EcR coregulators. For this purpose, an EGFP protein trap approach was applied to screen for factors colocalized with EcR on polytene chromosomes. Several candidate factors encoded known transcriptional coregulators: osa (a SWI/SNF complex component) (Supplemental Fig. S16) and skd (a mediator complex component) (Boube et al. 2000; Mohrmann et al. 2004; data not shown), as well as functionally uncharacterized proteins, including dDEK. The chromosomal localization revealed that dDEK supports the process of gene activation coupled to puff formation.

DEK is a histone chaperone

Two forms of DEK complexes were biochemically purified from insect and human cells, with very similar compositions (Figs. 3B, 7C, 7D; Supplemental Fig. S8B). We hypothesize that formation of the dDEK–CK2 complex is required for dDEK phosphorylation, rendering histone chaperone activity, while the dDEK–histone complex represents an intermediate during assembly into chromatin. Reflecting dDEK localization (Fig. 1C), histones associating with dDEK harbored epigenetic marks for transcriptional activation (Supplemental Fig. S12B). The purified complex contained H3.3 (data not shown), and dDEK appears to accommodate more H3.3–H4 tetramers than H3–H4 in S2 cells (Fig. 6A). Moreover, on polytene chromosomes, we observed that dDEK preferentially colocalized with H3.3 rather than with H3 (Fig. 6C). It is unclear at this stage if dDEK is also capable of disassembling histones from chromatin. While HIRA has been characterized as a histone chaperone for H3.3 (Tagami et al. 2004; Henikoff 2008), H3.3 deposition on chromosome has been observed in flies deficient of HIRA (Bonnefoy et al. 2007). dDEK may be functionally similar to HIRA in H3.3 assembly into chromatin. Together with the previous findings that nucleosomes containing H3.3 tend to be less stable than those containing H3 (Jin and Felsenfeld 2007), it is conceivable that dDEK remodels nucleosomal histone octamers into more transcriptionally active octamers through its histone chaperone activity, in cooperation with chromatin remodelers (Henikoff and Ahmad 2005; De Koning et al. 2007; Henikoff 2008). During puff formation, EcR may recruit dDEK to reconfigure the nucleosomal array during chromatin reorganization.

The histone chaperone activity of DEK depends on its phosphorylation by CK2

In the in vitro chromatin assembly system, DEK function required CK2, and a CK2 kinase inhibitor abrogated chromatin assembly. The dDEK mutant (GSK > AAA) was unable to stably associate with CK2α (Fig. 4B,C) and was defective in assembling histones on DNA (Fig. 5D). Thus, CK2 requires the phosphorylation of dDEK to form a dDEK–CK2 complex as a histone chaperone. Like CK2, the DEK–CK2 functional complex appears to be composed of two units: One is a phosphorylated dDEK tetramer functioning as a histone chaperone, and the other is a CK2 tetramer functioning as a regulatory unit. In this respect, DEK appears to be an atypical histone chaperone in terms of phosphorylation dependency as well as subunit composition, although a number of histone chaperones have been described with diverse forms of oligomerization (Park and Luger 2008).

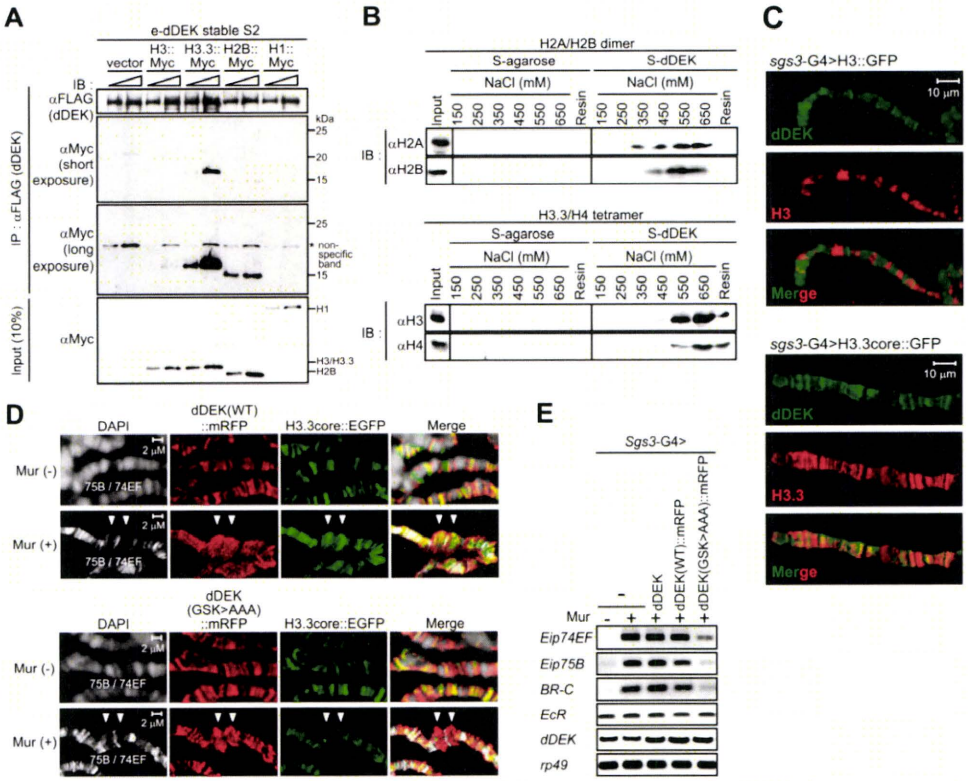

Figure 6. The nucleosomal assembly activity of dDEK is required for ecdysone-inducible puff formation and EcR target gene expression. (*A*) Preferential association of dDEK with a histone variant, H3.3. Chromatin fractions from e-dDEK stable cells transfected with H3-myc, H3.3-myc, H2B-myc, or H1-myc were subjected to immunoprecipitation using anti-Flag M2 resin. Each immunoprecipitated histone was detected using anti-Myc antibody. (*B*) Direct interaction of dDEK with histone H3.3/H4 tetramer. For pull-down assays, reconstituted recombinant *Drosophila* H2A/H2B dimer or H3.3/H4 tetramer were incubated with S-tagged dDEK protein immobilized on S-protein beads. Wash fractions (at each NaCl concentration) and final elution fractions were analyzed by Western blotting using the indicated antibodies. (*C*) Colocalization of dDEK with variant H3.3, but not canonical H3. Polytene chromosomes from larvae expressing H3::EGFP (*top*) or H3.3core::EGFP (*bottom*) fusion proteins driven by *sgs3-GAL4* were coimmunostained with anti-dDEK (green) and anti-GFP (red) antibodies. (*D*) dDEK-dependent accumulation of H3.3 at ecdysone-inducible puff loci 74EF and 75B (arrowheads). The salivary glands from larvae coexpressing H3.3core::EGFP (green) with dDEK(WT)::mRFP or dDEK(GSK > AAA)::mRFP (red) fusion proteins driven by *sgs3-GAL4* were treated without or with Mur (2.5×10^{-7} M) as described in Figure 1C. The polytene chromosomes were subjected to immunostaining with anti-GFP and anti-RFP antibodies. (*E*) Abrogated expression of EcR-target genes in the fly line expressing dDEK defective in histone chaperone activity. RT–PCR was performed in RNA isolated from larval salivary glands expressing dDEK, dDEK(WT)::mRFP, or dDEK(GSK > AAA)::mRFP transgene. Semiquantitative RT–PCR analysis was performed as described in Figure 2C.

DEK is a transcriptional coactivator for EcR

It is believed that histone chaperones coregulate transcription; however, no clear evidence, particularly in intact animals, supports a histone chaperone serving as a transcriptional coregulator for a given transcriptional activator. Here, we show that endogenous dDEK coactivates endogenous EcR in the chromosomal regions of ecdysone-induced puffs in intact flies (Fig. 2C). Its coactivator function is likely attributable to its histone chaperone activity (Fig. 6E; Supplemental Fig. S14), leading to assembly of active histones in chromatin (Fig. 6A; Supplemental Fig. S12B). As dDEK and EcR were detected together with CK2α at the EcR-binding sites in EcR target genes (Fig. 2E; Supplemental Fig. S5C), they likely contribute to ecdysone-dependent nucleosomal reorganization. In this respect, dDEK may be unique among histone chaperones in terms of its selective and physical interaction with EcR (Fig. 2A,B). However, other histone chaperones may also assist in the assembly and disassembly of histone octamers on promoter/enhancer sequences where EcR regulates gene expression. This concept is supported by data showing ecdysone-induced puff formation was not abrogated in a transgenic fly line overexpressing a dominant-negative form of dDEK

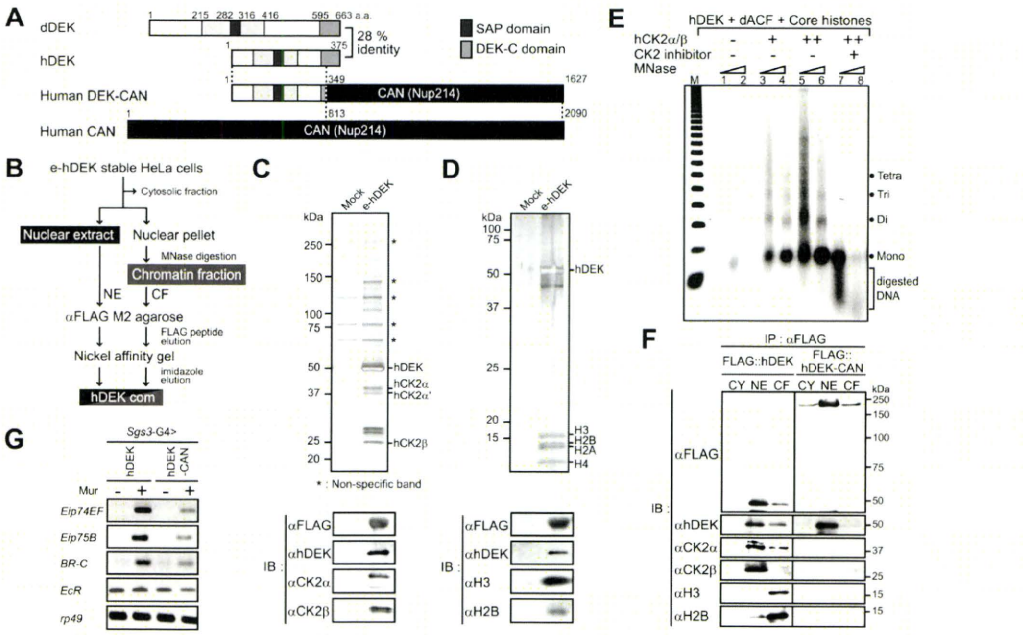

Figure 7. Functional characteristics of hDEK and leukemia-associated fusion protein, DEK-CAN. (*A*) Schematic representation of dDEK, hDEK, hCAN, and hDEK-CAN fusion proteins (von Lindern et al. 1992). (*B*) The purification scheme for the hDEK-containing complex. (*C*,*D*) Mass spectrometric and Western blot analysis of the hDEK complex components purified from the NE (*C*) or chromatin fraction (*D*) in e-hDEK stable HeLa cells. Silver staining of the peak fraction of hDEK from the final Ni affinity purification step is shown in the *top* panels. MALDI-TOF/MS analysis of the complex subunits is shown on the *right*. Asterisks indicate background proteins. Purified hDEK complex fractions were used for Western blotting with the indicated antibodies in the *bottom* panels. (*E*) Histone chaperone activity of hDEK in vitro. Recombinant hDEK was subjected to the nucleosome assembly assay as described in Figure 5B. (*F*) Lack of functional components of hDEK complex in hDEK-CAN-overexpressing 293T cells. The cytosolic fraction (CY), NE, and chromatin fraction (CF) derived from 293T cells transiently transfected with Flag-tagged hDEK or hDEK-CAN were used for immunoprecipitation using anti-Flag M2 resin. The immunoprecipitates were subjected to Western blotting with the indicated antibodies. (*G*) Abrogated expression of EcR target genes in the fly line expressing hDEK-CAN leukemic fusion protein. RT–PCR was performed in RNA isolated from larval salivary glands expressing hDEK or hDEK-CAN. Semiquantitative RT–PCR analysis was performed as described in Figure 2C.

(GSK > AAA) (Fig. 6D), regardless of aberrant expression of the tested EcR target genes (Fig. 6E).

dDEK localization on polytene chromosomes was also seen in regions other than the puff sites, and the localization pattern was not significantly affected by knockdown of EcR (data not shown). Since histone chaperone activity supports both eviction and assembly of histone octamers on chromatin, we speculate that the dDEK histone chaperone might serve as an inhibitor for transcriptional events, depending on dDEK localization on chromosomes. These facets of dDEK function may account for bidirectional coregulatory roles in transcriptional control; i.e., as an activator in the present study versus a repressor in a previous report (Gamble and Fisher 2007).

A class of AML is attributed to impaired histone chaperone activity of hDEK by fusion with CAN

The similarities of motif organizations in human and fly DEKs suggest that DEKs serve as a class of histone chaperone. Thus, it is feasible that this hDEK function is compromised by its fusion with CAN in AML patients (Fig. 7A; Soekarman et al. 1992; von Lindern et al. 1992). In the present study, we observed that the hDEK-CAN fusion protein is unable to associate with CK2 (Fig. 7F). As phosphorylation of DEK by CK2 was required for histone chaperone activity, it is most likely that histone chaperone function is impaired in the fusion protein. As this stage, the global roles of hDEK function as a histone chaperone in chromosomal changes remain to be defined. However, from the present observations, the hDEK-CAN fusion protein appears defective, at least in part, in assisting transcriptional regulation of certain genes that might be essential for normal differentiation of hematopoietic cells.

Materials and methods

For a detailed description of the Materials and Methods, see the Supplemental Material.

Screening

Salivary glands of third instar larvae of an EGFP protein trap line from 5-d-old cultures were dissected in phosphate-buffered saline (PBS). After removing as much fat as possible, the organ was cultured for 1 h at 22°C in 300 μL of *Drosophila* SFM (Invitrogen) containing 2.5×10^{-7} M Mur (Wako). After incubation, the glands were washed with PBS and immediately fixed for 20 min in 4% paraformaldehyde at room temperature. For immunofluorescence staining, they were incubated with anti-EcR primary antibodies (Ag10.2 and DDA2.7 from Developmental Studies Hybridoma Bank), then with Cy3-conjugated anti-mouse secondary antibody (Jackson ImmunoReserch) and DAPI (Roche) as a counterstain for 1 h at room temperature. Confocal microscopy was performed on a Zeiss Confocal Laser Scanning System 510, and images were assessed using Adobe Photoshop 7.0 (Adobe).

Immunostaining of polytene chromosome squashes

Immunostaining of polytene chromosome squashes from third instar larvae were performed as described in Zhao et al. (2009) with some modifications. Detailed information is available in the Supplemental Material.

Fly strains

Fly stocks were maintained at 22°C on standard cornmeal–agar–yeast food. The *yw* strain was used as wild-type in all experiments. *G00131* and *CA06616* (kind gifts from L. Cooley and A. Spradling) were generated by mobilizing the EGFP-trapping P-element as described (Morin et al. 2001; Buszczak et al. 2007). The fly lines expressing Flag-tagged or monomeric RFP (mRFP)-tagged dDEK, hDEK, hDEK-CAN, and dCK2α were created by transforming *Drosophila* germline cells (BestGene, Inc.) using the pUAST vectors. Several independent lines were established and crossed with *sgs3-GAL4* driver lines (*sgs3-G4*) expressed in salivary glands. RNAi transgenic fly lines of dDEK were obtained using the inducible RNAi method. A 661-base-pair (bp) cDNA fragment (nucleotide position 1–661 of the coding sequence) was amplified by PCR and inserted as an IR in the pUAST-R57 vector (provided by K. Saigo) (Pili-Floury et al. 2004). Knockdown flies were obtained by mating *sgs3-G4* to each of the UAS-IR lines. *UAS-IR-EcR* was obtained from Bloomington Stock Center. *UAS-IR-Tai* was provided from R. Ueda. *UAS-H3.3core::EGFP* and *UAS-H3::EGFP* lines were generous gifts from K. Ahmad (Schwartz and Ahmad 2005).

Purification of dDEK and hDEK complexes

Drosophila S2 cells stably expressing N-terminal Flag epitope-tagged DEK (e-dDEK) were established with a *Drosophila* Expression System (Invitrogen). The dDEK complex was immunoprecipitated from NEs (>0.1 g) with anti-Flag M2 resin in buffer D (20 mM HEPES-KOH, 0.2 mM EDTA, 5 mM $MgCl_2$, 150 mM KCl, 0.05% [v/v] NP-40, 10% [v/v] glycerol, 0.5 mM DTT, 0.2 mM PMSF, protease inhibitor cocktail [Roche] at pH 7.9). The bound polypeptides were eluted with the Flag peptide (0.2 mg/mL) and were subjected to Mono Q chromatography using AKTAexplorer 10S (GE Healthcare) (Fujiki et al. 2009). The components were identified by peptide mass fingerprint analysis using MALDI-TOF/MS (Bruker Daltonics) (Ohtake et al. 2007; Fujiki et al. 2009). For gel filtration, the fractions from the anti-Flag purification were loaded onto a Superose 6 10/300 GL column and fractionated with buffer D in 1-mL fractions using AKTAexplorer 10S.

Nucleosome assembly and MNase digestion assays

Nucleosome assembly and MNase digestion assays were performed as described previously (Ito et al. 1997; Kitagawa et al. 2003). A standard reaction contained supercoiled plasmid DNA, purified core histones from S2 cells, purified recombinant dDEK, purified recombinant dACF, ATP (3 mM), and the ATP-regenerating system.

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References

Adkins MW, Tyler JK. 2006. Transcriptional activators are dispensable for transcription in the absence of Spt6-mediated chromatin reassembly of promoter regions. *Mol Cell* **21:** 405–416.

Adkins MW, Howar SR, Tyler JK. 2004. Chromatin disassembly mediated by the histone chaperone Asf1 is essential for transcriptional activation of the yeast PHO5 and PHO8 genes. *Mol Cell* **14:** 657–666.

Ashburner M. 1967. Patterns of puffing activity in the salivary gland chromosomes of *Drosophila*. I. Autosomal puffing patterns in a laboratory stock of *Drosophila melanogaster*. *Chromosoma* **21:** 398–428.

Ashburner M. 1990. Puffs, genes, and hormones revisited. *Cell* **61:** 1–3.

Bai J, Uehara Y, Montell DJ. 2000. Regulation of invasive cell behavior by taiman, a *Drosophila* protein related to AIB1, a steroid receptor coactivator amplified in breast cancer. *Cell* **103:** 1047–1058.

Bonnefoy E, Orsi GA, Couble P, Loppin B. 2007. The essential role of *Drosophila* HIRA for de novo assembly of paternal chromatin at fertilization. *PLoS Genet* **3:** 1991–2006.

Borrelli E, Nestler EJ, Allis CD, Sassone-Corsi P. 2008. Decoding the epigenetic language of neuronal plasticity. *Neuron* **60:** 961–974.

Boube M, Faucher C, Joulia L, Cribbs DL, Bourbon HM. 2000. *Drosophila* homologs of transcriptional mediator complex subunits are required for adult cell and segment identity specification. *Genes & Dev* **14:** 2906–2917.

Burtis KC, Thummel CS, Jones CW, Karim FD, Hogness DS. 1990. The *Drosophila* 74EF early puff contains E74, a complex ecdysone-inducible gene that encodes two ets-related proteins. *Cell* **61:** 85–99.

Buszczak M, Paterno S, Lighthouse D, Bachman J, Planck J, Owen S, Skora AD, Nystul TG, Ohlstein B, Allen A, et al. 2007. The carnegie protein trap library: A versatile tool for *Drosophila* developmental studies. *Genetics* **175:** 1505–1531.

Cairns BR. 2009. The logic of chromatin architecture and remodelling at promoters. *Nature* **461:** 193–198.

De Koning L, Corpet A, Haber JE, Almouzni G. 2007. Histone chaperones: An escort network regulating histone traffic. *Nat Struct Mol Biol* **14:** 997–1007.

Evans RM. 1988. The steroid and thyroid hormone receptor superfamily. *Science* **240:** 889–895.

Fujiki R, Chikanishi T, Hashiba W, Ito H, Takada I, Roeder RG, Kitagawa H, Kato S. 2009. GlcNAcylation of a histone methyltransferase in retinoic-acid-induced granulopoiesis. *Nature* **459:** 455–459.

Gamble MJ, Fisher RP. 2007. SET and PARP1 remove DEK from chromatin to permit access by the transcription machinery. *Nat Struct Mol Biol* **14:** 548–555.

Green S, Chambon P. 1988. Nuclear receptors enhance our understanding of transcription regulation. *Trends Genet* **4:** 309–314.

Henikoff S. 2008. Nucleosome destabilization in the epigenetic regulation of gene expression. *Nat Rev Genet* **9:** 15–26.

Henikoff S, Ahmad K. 2005. Assembly of variant histones into chromatin. *Annu Rev Cell Dev Biol* **21:** 133–153.

Ito T, Bulger M, Pazin MJ, Kobayashi R, Kadonaga JT. 1997. ACF, an ISWI-containing and ATP-utilizing chromatin assembly and remodeling factor. *Cell* **90:** 145–155.

Jiang C, Pugh BF. 2009. Nucleosome positioning and gene regulation: Advances through genomics. *Nat Rev Genet* **10:** 161–172.

Jin C, Felsenfeld G. 2007. Nucleosome stability mediated by histone variants H3.3 and H2A.Z. *Genes & Dev* **21:** 1519–1529.

Karim FD, Thummel CS. 1992. Temporal coordination of regulatory gene expression by the steroid hormone ecdysone. *EMBO J* **11:** 4083–4093.

Kim MY, Mauro S, Gevry N, Lis JT, Kraus WL. 2004. NAD+-dependent modulation of chromatin structure and transcription by nucleosome binding properties of PARP-1. *Cell* **119:** 803–814.

King-Jones K, Thummel CS. 2005. Nuclear receptors—a perspective from *Drosophila*. *Nat Rev Genet* **6:** 311–323.

Kitagawa H, Fujiki R, Yoshimura K, Mezaki Y, Uematsu Y, Matsui D, Ogawa S, Unno K, Okubo M, Tokita A, et al. 2003. The chromatin-remodeling complex WINAC targets a nuclear receptor to promoters and is impaired in Williams syndrome. *Cell* **113:** 905–917.

Koelle MR, Talbot WS, Segraves WA, Bender MT, Cherbas P, Hogness DS. 1991. The *Drosophila* EcR gene encodes an ecdysone receptor, a new member of the steroid receptor superfamily. *Cell* **67:** 59–77.

Kouzarides T. 2007. Chromatin modifications and their function. *Cell* **128:** 693–705.

Litchfield DW. 2003. Protein kinase CK2: Structure, regulation and role in cellular decisions of life and death. *Biochem J* **369:** 1–15.

Mito Y, Henikoff JG, Henikoff S. 2005. Genome-scale profiling of histone H3.3 replacement patterns. *Nat Genet* **37:** 1090–1097.

Mohrmann L, Langenberg K, Krijgsveld J, Kal AJ, Heck AJ, Verrijzer CP. 2004. Differential targeting of two distinct SWI/SNF-related *Drosophila* chromatin-remodeling complexes. *Mol Cell Biol* **24:** 3077–3088.

Morin X, Daneman R, Zavortink M, Chia W. 2001. A protein trap strategy to detect GFP-tagged proteins expressed from their endogenous loci in *Drosophila*. *Proc Natl Acad Sci* **98:** 15050–15055.

Morrison AJ, Shen X. 2009. Chromatin remodelling beyond transcription: The INO80 and SWR1 complexes. *Nat Rev Mol Cell Biol* **10:** 373–384.

Narlikar GJ, Fan HY, Kingston RE. 2002. Cooperation between complexes that regulate chromatin structure and transcription. *Cell* **108:** 475–487.

Ohtake F, Baba A, Takada I, Okada M, Iwasaki K, Miki H, Takahashi S, Kouzmenko A, Nohara K, Chiba T, et al. 2007. Dioxin receptor is a ligand-dependent E3 ubiquitin ligase. *Nature* **446:** 562–566.

Park YJ, Luger K. 2008. Histone chaperones in nucleosome eviction and histone exchange. *Curr Opin Struct Biol* **18:** 282–289.

Pili-Floury S, Leulier F, Takahashi K, Saigo K, Samain E, Ueda R, Lemaitre B. 2004. In vivo RNA interference analysis reveals an unexpected role for GNBP1 in the defense against Gram-positive bacterial infection in *Drosophila* adults. *J Biol Chem* **279:** 12848–12853.

Roeder RG. 2005. Transcriptional regulation and the role of diverse coactivators in animal cells. *FEBS Lett* **579:** 909–915.

Rosenfeld MG, Lunyak VV, Glass CK. 2006. Sensors and signals: A coactivator/corepressor/epigenetic code for integrating signal-dependent programs of transcriptional response. *Genes & Dev* **20:** 1405–1428.

Sawatsubashi S, Maki A, Ito S, Shirode Y, Suzuki E, Zhao Y, Yamagata K, Kouzmenko A, Takeyama K, Kato S. 2004. Ecdysone receptor-dependent gene regulation mediates histone poly(ADP-ribosyl)ation. *Biochem Biophys Res Commun* **320:** 268–272.

Schwartz BE, Ahmad K. 2005. Transcriptional activation triggers deposition and removal of the histone variant H3.3. *Genes & Dev* **19:** 804–814.

Sedkov Y, Cho E, Petruk S, Cherbas L, Smith ST, Jones RS, Cherbas P, Canaani E, Jaynes JB, Mazo A. 2003. Methylation at lysine 4 of histone H3 in ecdysone-dependent development of *Drosophila*. *Nature* **426:** 78–83.

Sims RJ III, Reinberg D. 2008. Is there a code embedded in proteins that is based on post-translational modifications? *Nat Rev Mol Cell Biol* **9:** 815–820.

Soekarman D, von Lindern M, Daenen S, de Jong B, Fonatsch C, Heinze B, Bartram C, Hagemeijer A, Grosveld G. 1992. The translocation (6;9) (p23;q34) shows consistent rearrangement of two genes and defines a myeloproliferative disorder with specific clinical features. *Blood* **79:** 2990–2997.

Stabell M, Larsson J, Aalen RB, Lambertsson A. 2007. *Drosophila* dSet2 functions in H3-K36 methylation and is required for development. *Biochem Biophys Res Commun* **359:** 784–789.

Strahl BD, Allis CD. 2000. The language of covalent histone modifications. *Nature* **403:** 41–45.

Tagami H, Ray-Gallet D, Almouzni G, Nakatani Y. 2004. Histone H3.1 and H3.3 complexes mediate nucleosome assembly pathways dependent or independent of DNA synthesis. *Cell* **116:** 51–61.

Takeyama K, Ito S, Yamamoto A, Tanimoto H, Furutani T, Kanuka H, Miura M, Tabata T, Kato S. 2002. Androgen-dependent neurodegeneration by polyglutamine-expanded human androgen receptor in *Drosophila*. *Neuron* **35:** 855–864.

Thummel CS. 1996. Files on steroids—*Drosophila* metamorphosis and the mechanisms of steroid hormone action. *Trends Genet* **12:** 306–310.

Thummel CS. 2002. Ecdysone-regulated puff genes 2000. *Insect Biochem Mol Biol* **32:** 113–120.

von Lindern M, Fornerod M, van Baal S, Jaegle M, de Wit T, Buijs A, Grosveld G. 1992. The translocation (6;9), associated with a specific subtype of acute myeloid leukemia, results in the fusion of two genes, dek and can, and the expression of

a chimeric, leukemia-specific dek-can mRNA. *Mol Cell Biol* **12:** 1687–1697.

Weeks JR, Hardin SE, Shen J, Lee JM, Greenleaf AL. 1993. Locus-specific variation in phosphorylation state of RNA polymerase II in vivo: Correlations with gene activity and transcript processing. *Genes & Dev* **7:** 2329–2344.

Wittig I, Braun HP, Schagger H. 2006. Blue native PAGE. *Nat Protoc* **1:** 418–428.

Zhao Y, Takeyama K, Sawatsubashi S, Ito S, Suzuki E, Yamagata K, Tanabe M, Kimura S, Fujiyama S, Ueda T, et al. 2009. Corepressive action of CBP on androgen receptor transactivation in pericentric heterochromatin in a *Drosophila* experimental model system. *Mol Cell Biol* **29:** 1017–1034.

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Idiopathic hypereosinophilic syndrome in a case with ABO-incompatible liver transplantation for biliary atresia complicated by portal vein thrombosis

Yamada Y, Hoshino K, Shimojima N, Shinoda M, Obara H, Kawachi S, Fuchimoto Y, Tanabe M, Kitagawa Y, Morikawa Y. Idiopathic hypereosinophilic syndrome in a case with ABO-incompatible liver transplantation for biliary atresia complicated by portal vein thrombosis.
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Abstract: Idiopathic HES is characterlized by prolonged eosinophilia without an identifiable underlying cause and multiple-organ dysfunction. We report a case of a LDLT for a 12-yr-old Japanese girl with BA accompanied by HES. Histological examination of the resected liver showed biliary cirrhosis with dense eosinophilic infiltration of portal tracts and the lobules of the liver. She developed portal vein thrombosis on post-operative day 10 and the histopathological findings of the thrombus revealed dense eosinophilic deposition, suggesting that HES might have influenced the formation of this thrombus. Liver graft biopsies also demonstrated the presence of activated eosinophilils with biliary damage. Blood chemistry findings suggested liver dysfunction as a result of the eosinophilic infiltrations. Prednisolone treatment improved the liver dysfunction. Four years after LDLT, she remains clinically well on prednisolone at 0.3 mg/kg/day, with an eosinophil count ranging from 10 to 15%. A literature review has not shown any previous reports of HES with BA. This case demonstrates the possibility of an association between eosinophilic infiltration and liver dysfunction during follow-up for BA and after LDLT.

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HES is a heterogeneous group of disorders, characterized by prolonged eosinophilia without an identifiable cause, and multiple organ dysfunction involving the heart, the central or peripheral nervous system, lungs, liver, and gastrointestinal tract. The diagnosis of HES has been one of exclusion because the pathophysiology of the syndrome is largely unknown. Because of its low prevalence, few reports have demonstrated organ transplantation with HES. We describe a 12-yr-old Japanese girl with BA accompanied by HES who received LDLT. She developed portal vein thrombosis and hepatitis possibly because of HES post-operatively and was subsequently successfully managed by thrombectomy and steroids. In this paper, we report perioperative complications and management.

Abbreviations: ALT, alanine aminotransferase; aPTT, activated partial thromboplastin time; AST, aspartate aminotransferase; BA, biliary atresia; CT, computed tomography; ECP, eosinophil cationic protein; GM-CSF, granulocyte-macrophage colony stimulating factor; HES, hypereosinophilic syndrome; LDLT, living donor liver transplantation; NCC, nuclear cell count; POD, preoperative days; POM, preoperative months; PSC, primary sclerosing cholangitis; TB, total bilirubin.

Case study

Patient is a 12-yr-old Japanese girl. She was the product of a full-term normal delivery. She was noted to have jaundice at a routine health