in vitro system (*3*). Acetylation-dependent accessibility may therefore require more-complex chromosomal substrates or remodeling activities that are present in vivo but not yet reproduced in vitro. We suggest a model for V(D)J recombination in which cis-regulatory elements direct access to RAG proteins in vivo by inducing the region- and developmental stage–specific hyperacetylation of histone H3.

### References and Notes


1. M. Gellert, *Adv. Immunol.* **64**, 39 (1997); D. G. Schatz, *Sem. Immunol.* **9**, 149 (1997); U. Grawunder, R. B. West, M. R. Lieber, *Curr. Opin. Immunol.* **10**, 172 (1998).
2. P. Stanhope-Baker, K. M. Hudson, A. L. Shaffer, A. Constantinescu, M. S. Schlissel, *Cell* **85**, 887 (1996); J. Kwon, A. N. Imbalzano, A. Matthews, M. A. Oettinger, *Mol. Cell* **2**, 829 (1998).
3. A. Golding, S. Chandler, E. Ballestar, A. P. Wolffe, M. S. Schlissel, *EMBO J.* **18**, 3712 (1999).
4. B. P. Sleckman, J. R. Gorman, F. W. Alt, *Annu. Rev. Immunol.* **14**, 459 (1996); M. S. Schlissel and P. Stanhope-Baker, *Sem. Immunol.* **9**, 161 (1997); W. M. Hempel, I. Leduc, N. Mathieu, R. K. Tripathi, P. Ferrier, *Adv. Immunol.* **69**, 309 (1998).
5. P. L. Sheridan, T. P. Mayall, E. Verdin, K. A. Jones, *Genes Dev.* **11**, 3327 (1998); L. Madisen, A. Krumm, T. R. Hebbes, M. Groudine, *Mol. Cell. Biol.* **18**, 6281 (1998); M.-H. Kuo, J. Zhou, P. Jambeck, M. E. A. Churchill, C. D. Allis, *Genes Dev.* **12**, 627 (1998); B. S. Parekh and T. Maniatis, *Mol. Cell* **3**, 125 (1999).
6. A. P. Wolffe, J. Wong, D. Pruss, *Genes Cells* **2**, 291 (1997); K. Struhl, *Genes Dev.* **12**, 599 (1998); J. L. Workman and R. E. Kingston, *Annu. Rev. Biochem.* **67**, 545 (1998).
7. T. R. Hebbes, A. L. Clayton, A. W. Thorne, C. Crane-Robinson, *EMBO J.* **13**, 1823 (1994).
8. D. Lee, J. J. Hayes, D. Pruss, A. P. Wolffe, *Cell* **72**, 73 (1993); M. Vettese-Dadey *et al.*, *EMBO J.* **15**, 2508 (1996).
9. P. Lauzurica and M. S. Krangel, *J. Exp. Med.* **179**, 43 (1994).
10. ———, *J. Exp. Med.* **179**, 1913 (1994).
11. C. Hernandez-Munain, P. Lauzurica, M. S. Krangel, *J. Exp. Med.* **183**, 289 (1996).
12. P. Lauzurica, X.-P. Zhong, M. S. Krangel, J. L. Roberts, *J. Exp. Med.* **185**, 1193 (1997).
13. M. T. McMurry, C. Hernandez-Munain, P. Lauzurica, M. S. Krangel, *Mol. Cell. Biol.* **17**, 4553 (1997).
14. Mononucleosomes were prepared from thymocytes of 4- to 8-week-old mice with modifications from Hebbes *et al.* (*7*). Thymocytes ($1 \times 10^9$ to $4 \times 10^9$) were filtered through nylon mesh, centrifuged (350*g*, 7 min), and the pellet resuspended at $4 \times 10^7$ cells/ml in 0.15 M $NH_4Cl$, 1 mM $KHCO_3$, 0.1 mM $Na_2EDTA$, 10 mM sodium butyrate, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.1 mM benzamidine (pH 7.4) for 5 min at 23°C to lyse red blood cells. Thymocytes were then washed twice and resuspended at $1 \times 10^8$/ml in lysis buffer [80 mM NaCl, 10 mM tris-HCl (pH 7.5), 10 mM sodium butyrate, 6 mM $MgCl_2$, 1 mM $CaCl_2$, 250 mM sucrose, 0.1 mM PMSF, and 0.1 mM benzamidine], and an equal volume of buffer containing 0.04% (v/v) NP-40 was added for 5 min at 4°C. Nuclei were pelleted through 30% sucrose by centrifugation at 350*g* for 7 to 14 min and washed once, and were then resuspended in digestion buffer at 5 mg/ml ($7 \times 10^8$ nuclei/ml) by absorbance at 260 nm. Nuclei were incubated with 1000 U/ml micrococcal nuclease (Worthington, Freehold, NJ) for 10 min at 37°C to produce a partial digest (*25*). After shift to 0°C and addition of $Na_3EDTA$ to 5 mM, the digest was centrifuged at 13,000*g* for 1 min at 4°C, the pellet was washed, and supernatants from the two centrifugations were combined and NaCl added to 50 mM. Histone H1 was removed by incubation for 1.5 hours at 4°C with 30 mg/ml Sephadex CM-25, followed by centrifugation for 5 min at 13,000*g*. A Biocomp Gradient Mate (Accurate Chemical and Scientific, Westbury, NY) was used to prepare 14 ml of 10 to 45% linear sucrose gradients in 10 mM tris-HCl (pH 7.5), 0.25 mM $Na_3EDTA$ (pH7.0), 10 mM sodium butyrate, 100 mM NaCl, 0.1 mM PMSF, and 0.1 mM benzamidine, and 2 to 3 mg of H1-depleted, digested chromatin was loaded onto each gradient and centrifuged for 18 hours at 40,000 rpm in an SW-40 rotor (Beckman Instruments, Fullerton, CA) at 4°C. Mononucleosome-containing fractions were identified as described (*26*). Immunoprecipitation of freshly prepared mononucleosomes (25 to 40 μg) was performed as described (*7*), with modifications. Pooled gradient fractions were first treated with 0.6 μl of 50% salmon sperm DNA–protein A-Sepharose slurry per μg of mononucleosomes for 30 min at 4°C, followed by centrifugation for 5 min at 13,000*g*. The supernatant was incubated with antibody to diacetylated histone H3–purified rabbit immunoglobulin G (IgG) (Upstate Biotechnology, Lake Placid NY) or control rabbit IgG (Sigma) (0.25 μg/μg of DNA) for 16 hours, followed by addition of salmon sperm DNA–protein A-Sepharose (12 μl/μg of antibody) for 1 hour. After centrifugation, the supernatant was retained, the IP washed, and DNA purified from these and input fractions (*7*).
15. Serial threefold dilutions (60, 20, and 6.6 ng) of bound, unbound, and input DNA fractions were analyzed by 25 cycles of PCR (45 s at 94°C, 1 min at 55°C or 59°C, and 2 min at 72°C) in 25-μl reactions as described (*13*). A single serial dilution series produced from each DNA fraction was typically used for PCR reactions with all primer sets. Primers and probes are listed in Table 1. Primer pairs were screened initially for their ability to amplify pure mononucleosomal DNA as efficiently as unfractionated genomic DNA. PCR products (which ranged in size from 99 to 150 bp) were subjected to electrophoresis through 3% Nusieve 3:1 agarose gels (FMC Bioproducts, Rockland, ME) and transferred to Magna Charge nylon membranes (0.22 μm; Osmonics, Westborough, MA). Probes were $^{32}P$-labeled by random priming.
16. C. Hernandez-Munain, B. P. Sleckman, M. S. Krangel, *Immunity* **10**, 723 (1999).
17. Y. Shinkai *et al.*, *Cell* **68**, 855 (1992).
18. Acetylation was quantified by PhophorImager (Molecular Dynamics) analysis of Southern blots. Values of B/U are reported, where B is the bound fraction of the αAcH3 IP and U is the unbound fraction of the control IP. The latter was used instead of the unbound fraction of the αAcH3 IP to prevent the ratios from being artificially inflated by depletion of the unbound fraction in instances of heavy acetylation. B/U ratios were calculated by determining the displacement between the B and U titration curves.
19. Y. Shinkai *et al.*, *Science* **259**, 822 (1993).
20. M. S. Krangel *et al.*, *Immunol. Rev.* **165**, 131 (1998).
21. R. J. Monroe *et al.*, *Immunity* **10**, 503 (1999).
22. B. P. Sleckman, C. G. Bardon, R. Ferrini, L. Davidson, F. W. Alt, *Immunity* **7**, 505 (1997).
23. M. T. McMurry and M. S. Krangel, unpublished observations.
24. X.-P. Zhong and M. S. Krangel, *Proc. Natl. Acad. Sci. U.S.A.* **94**, 5219 (1997).
25. Southern blot analysis of titrated nuclease digests indicated that the sensitivity to nuclease digestion of analyzed sequences was similar to that of bulk DNA.
26. J. J. Hayes and K.-M. Lee, *Methods* **12**, 2 (1997).
27. The RAG-$2^{-/-}$ sample is actually R×A, but is designated RAG-$2^{-/-}$ for clarity because the endogenous locus rather than the transgene is analyzed. The *Oct-2* panel for this sample is identical to that for R×A in Fig. 2B.


28. We thank B. Sleckman, Y. Zhuang, and C. Doyle for comments on the manuscript and encouragement, and C. Hernandez-Munain for advice. Supported by NIH grant GM 41052. M.T.M. was supported in part by an NIH Medical Scientist Training Grant and a United Negro College Fund–Merck Graduate Science Research Dissertation Fellowship.

16 September 1999; accepted 24 November 1999


# A Critical Role for Murine Complement Regulator Crry in Fetomaternal Tolerance


**Chenguang Xu,[1]* Dailing Mao,[1]* V. Michael Holers,[2] Ben Palanca,[1] Alec M. Cheng,[1] Hector Molina[1,3]†**



**Complement is a component of natural immunity. Its regulation is needed to protect tissues from inflammation, but mice with a disrupted gene for the complement regulator decay accelerating factor were normal. Mice that were deficient in another murine complement regulator, Crry, were generated to investigate its role in vivo. Survival of *Crry*$^{-/-}$ embryos was compromised because of complement deposition and concomitant placenta inflammation. Complement activation at the fetomaternal interface caused the fetal loss because breeding to *C3*$^{-/-}$ mice rescued *Crry*$^{-/-}$ mice from lethality. Thus, the regulation of complement is critical in fetal control of maternal processes that mediate tissue damage.**


Activation of complement promotes natural immunity by inducing chemotaxis of inflammatory cells, enhancing phagocytosis by neutrophils and monocytes, facilitating immune complex clearance, and mediating cell lysis by the membrane attack complex (*1*). Complement can also bind and attack self tissues, especially in areas of active inflammation. In vitro studies have shown that cells are protected from the deleterious effects of complement by proteins that regulate complement activation (*2*).

Three membrane-bound proteins regulate the activation of the third and fourth components of complement (C3 and C4) on the


[1]Departments of Medicine and Pathology, Washington University School of Medicine, St. Louis, MO 63110, USA. [2]Departments of Medicine and Immunology, University of Colorado Health Science Center, Denver, CO 80262, USA. [3]Veteran's Administration Medical Center, St. Louis, MO 63106, USA.

*These authors contributed equally to this work.
†To whom correspondence should be addressed. E-mail: hmolina@imgate.wustl.edu

surface of murine and human cells (*3*). Decay accelerating factor (DAF) inactivates the C3 convertase enzymes that activate C3. Membrane cofactor protein (MCP) serves as a cofactor for factor I–mediated degradation of activated C3 and C4. Crry, present only in rodents (*4–6*), regulates the deposition of activated C3 and C4 on the surface of autologous cells in vitro by exhibiting MCP- and DAF-like activities, although its relative contribution to complement regulation as compared to mouse MCP and DAF has not been elucidated.

Although decreased expression of complement regulatory molecules has been found in different inflammatory disorders (*7*), their specific contribution to pathogenesis is largely unknown. To investigate the role of complement regulation in vivo, we generated mice deficient in Crry by inserting a neomycin resistance gene that disrupted exon 5 of the mouse *Crry* gene in embryonal stem (ES) cells (*8, 9*). Three targeted ES cell clones with the expected homologous recombination were identified by Southern blotting (*10*). Two independently isolated cell clones were used to generate chimeric mice that subsequently transmitted the mutant allele to their progeny. Heterozygous germ line mutants appeared healthy and fertile.

Heterozygous animals were intercrossed to generate *Crry* null mice. However, no $Crry^{-/-}$ mice could be recovered from a total of 245 births, indicating that Crry deficiency resulted in embryonic lethality (Table 1). To determine the stage of lethality, we collected and genotyped embryos at various stages of development. At 9.5 days post coitus (dpc) or earlier, embryos with the expected frequency of the homozygous mutation were detected (~25%). In contrast, the percentage of homozygous mutants declined progressively thereafter. In addition, most $Crry^{-/-}$ embryos at 9.5 dpc had signs of developmental arrest, such as the smaller deciduae resembling those of earlier stages (Fig. 1, A and B). Decidua dissection revealed developmentally arrested, and sometimes deceased, embryos (Fig. 1, D through J). To confirm that the targeted mutation was a null allele, we studied protein expression from primary fibroblasts prepared from 13.5-dpc Crry-deficient embryos. In contrast to the wild-type control, staining with a Crry-specific antibody revealed that these $Crry^{-/-}$ fibroblasts did not express detectable Crry protein as determined by flow cytometry (*8, 11*). The insertional mutation therefore behaves as a null allele. These results suggest that Crry plays a crucial role during early embryonic development.

To determine the role of Crry on this developmental defect, we first analyzed its expression pattern in wild-type early embryos. Immunohistochemical detection of Crry in cryosectioned embryos indicated that Crry is highly expressed in trophoblasts as early as 7.5 dpc, with little expression in the embryo proper (*12*). In addition, Crry is also expressed in the maternally derived decidual tissues (Fig. 2A). This expression pattern persists in later stages of embryonic development (examined up to 16 dpc) (*13*). As expected, there is no Crry expression in $Crry^{-/-}$ trophoblast and embryos (Fig. 2B).

Given that Crry has been implicated as a negative regulator of complement activation

**Table 1.** Genotype analysis of littermates from $Crry^{+/-}$ matings. Numbers in parentheses represent the percentage of the total.

| Age | $Crry^{+/+}$ | $Crry^{+/-}$ | $Crry^{-/-}$ | Total |
|---|---|---|---|---|
| 3 weeks old | 113 (46) | 133 (54) | 0 (0) | 246 |
| 16.5 dpc | 5 (56) | 4 (44) | 0 (0) | 9 |
| 13.5 dpc | 19 (31) | 40 (64) | 3 (5) | 62 |
| 11.5 dpc | 22 (33) | 40 (61) | 4 (6) | 66 |
| 10.5 dpc | 21 (40) | 26 (50) | 5 (10) | 52 |
| 9.5 dpc | 25 (28) | 44 (49) | 21 (23) | 90 |

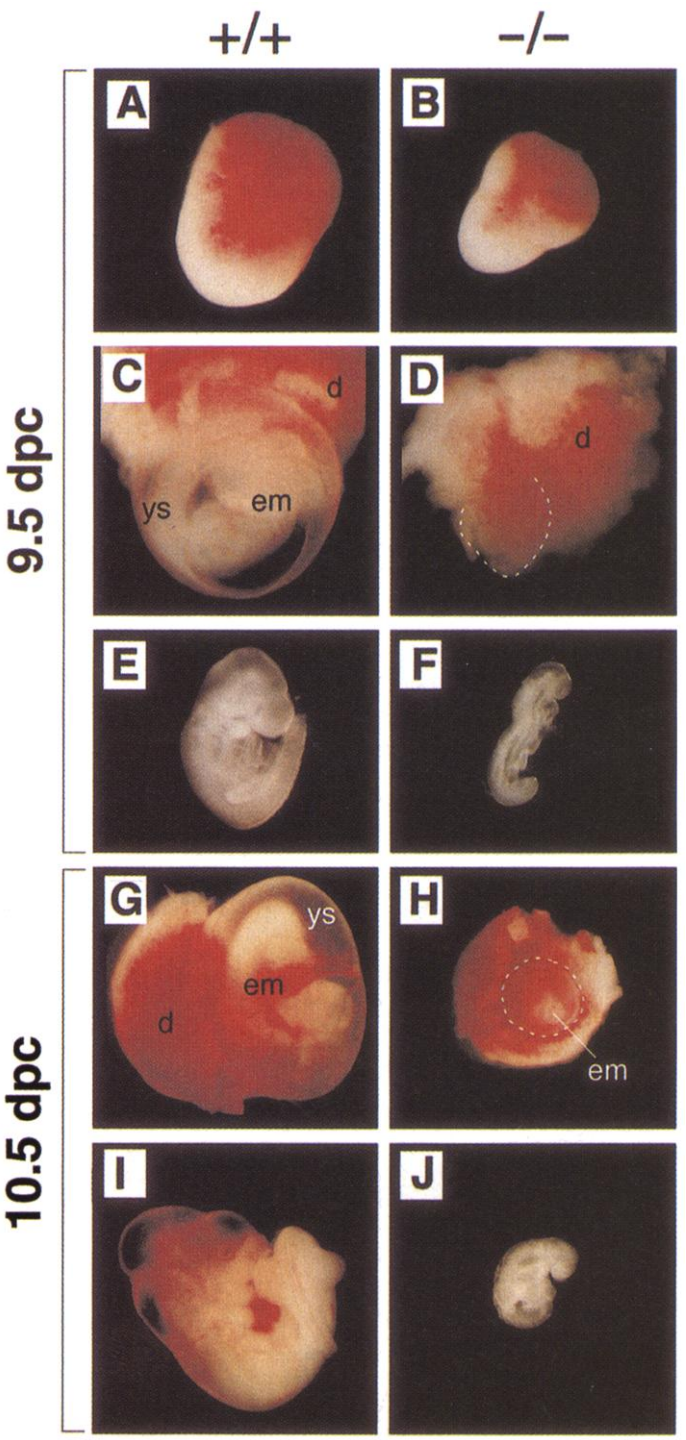


**Fig. 1.** Developmental arrest of $Crry^{-/-}$ embryos. $Crry^{+/+}$ (left panels) and $Crry^{-/-}$ (right panels) embryos. (**A** and **B**) Whole deciduae from 9.5-dpc embryos, ×1 magnification. (**C** and **D**) Decidua dissection to expose the yolk sac containing a 9.5-dpc embryo, ×2 magnification. (**E** and **F**) A 9.5-dpc embryo, ×2 magnification. (**G** and **H**) Decidua dissection to expose the yolk sac containing a 10.5-dpc embryo, ×1 magnification. (**I** and **J**) A 10.5-dpc embryo, ×2 magnification. d, maternal decidua; ys, yolk sac [dashed areas in (D) and (H)]; em, embryos.

and because we demonstrated that it is normally expressed in embryonic tissue, we hypothesized that the developing $Crry^{-/-}$ embryos died from their inability to suppress spontaneous complement activation and tissue inflammation in the areas around the decidua and trophoectoderm. To test this hypothesis, we compared the state of C3 activation on $Crry^{+/+}$ and $Crry^{-/-}$ embryos by staining with an antibody to mouse C3. In principle, native C3 is only present in soluble form, whereas activated C3 binds to the cell surface. In contrast to wild-type embryos (Fig. 2C), $Crry^{-/-}$ embryos had surface-deposited C3 in the trophoectoderm and the ectoplacental cone. Thus, the lack of Crry was associated with abnormal activation and deposition of complement (Fig. 2D).

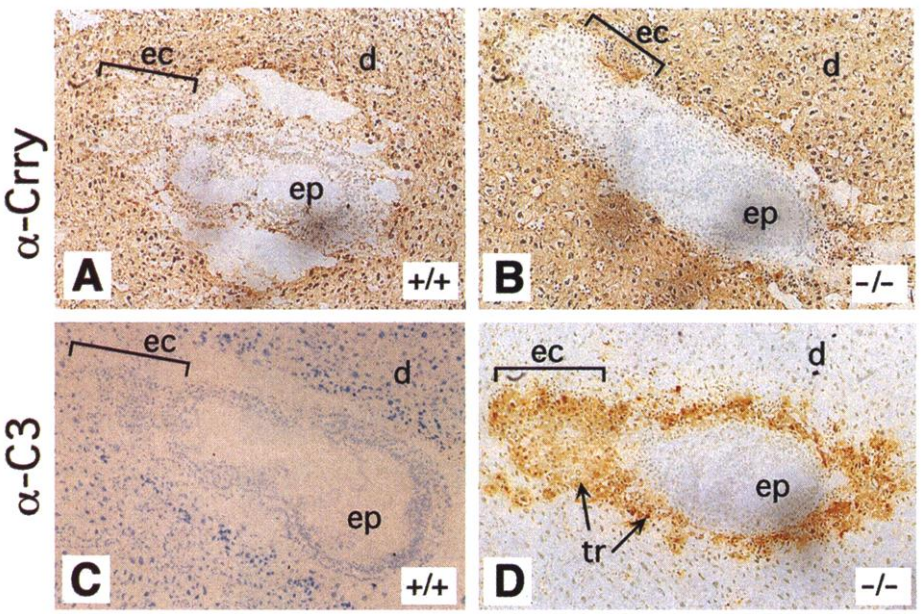


**Fig. 2.** Spontaneous complement activation in Crry-deficient embryos (×10 magnification). Staining of (**A**) $Crry^{+/+}$ and (**B**) $Crry^{-/-}$ 7.5-dpc embryos with a rabbit antibody to mouse Crry (α-Crry) or (**C**) $Crry^{+/+}$ and (**D**) $Crry^{-/-}$ embryos with an antibody to mouse C3 (α-C3). d, maternal decidua; ec, ectoplacental cone; ep, embryo proper; tr, trophoectoderm.

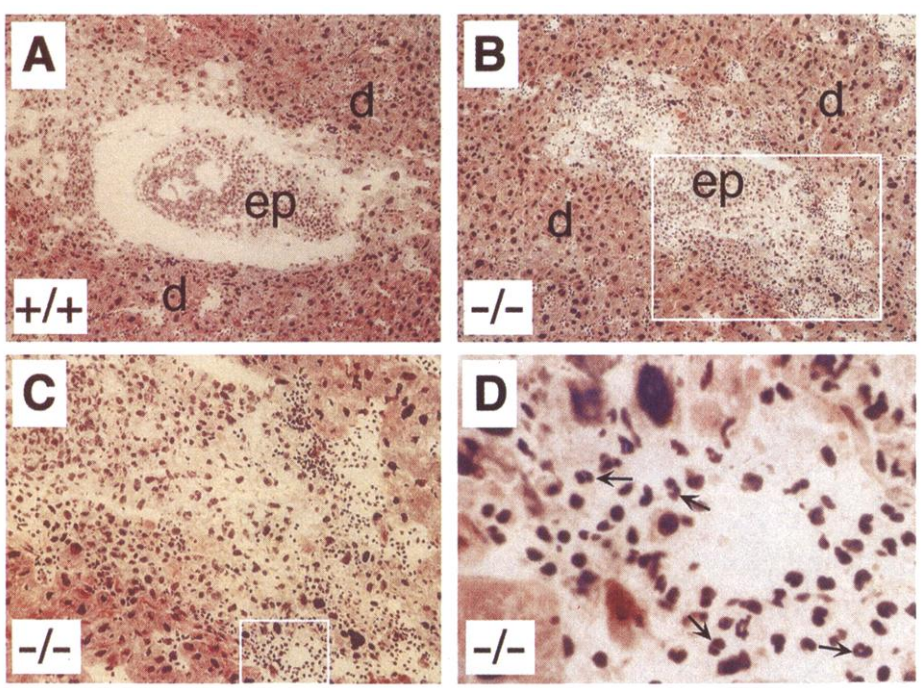


**Fig. 3.** Infiltration of polymorphonuclear cells in the extraembryonic tissues of the Crry-deficient embryos. (**A**) $Crry^{+/+}$ embryo and (**B**) $Crry^{-/-}$ embryo at ×10 magnification. (**C**) A ×40 magnification of the boxed area in (B). (**D**) A ×100 magnification of the boxed area in (C). Arrows denote polymorphonuclear cells. d, maternal decidua; ep, embryo proper.

To test if the spontaneous activation of C3 was the major mechanism by which embryonic lethality is observed in the $Crry^{-/-}$ mice, we examined the effect of this mutation in a C3-deficient background (*14*). To this end, we generated compound mutant mice that were $Crry^{+/-}$ and $C3^{-/-}$ and subsequently intercrossed them to generate mutants that were either Crry-sufficient or Crry-deficient in the C3-deficient background. Genotype analysis revealed that 27% (11 out of 41) of the resulting 3-week-old pups were Crry-deficient mutants, in contrast to the absence of $Crry^{-/-}$ newborns derived from the crossing of $C3^{+/+}Crry^{+/-}$ mice (Table 1), indicating that the $Crry^{-/-}$ embryonic lethality results from the activation of complement (*15*).

One potential consequence of complement activation is the establishment of an inflammatory reaction in the target tissue due to the recruitment and activation of granulocytes (*2*). To investigate if the absence of Crry initiated a similar reaction, we examined histological sections of 7.5- and 8.5-dpc embryos. In contrast to wild-type controls, $Crry^{-/-}$ embryos were extensively invaded by polymorphonuclear inflammatory granulocytes in areas around the ectoplacental cone and the surrounding trophoectoderm (Fig. 3). Thus, failure to control complement activation leads to an inflammatory response in the $Crry^{-/-}$ fetuses and, eventually, to embryonic demise.

It has been suggested that complement may be important in the reproductive system and in pregnancy (*16*). Our data indicate that complement regulation is indeed important in fetoplacental survival, maintenance of normal pregnancy, and adequate reproductive function by maintaining a form of fetomaternal tolerance against immunological mechanisms of tissue damage related to natural immunity.

Our studies are also relevant to the possible involvement of these molecules in pathologic pregnancies, both in animals and in humans, in which complement is believed to be involved in the disease pathogenesis (*17*). Given that mouse Crry and human DAF and MCP control C3 activation by the same biochemical mechanisms in vitro (*4–6*), we provide insight into the roles of the corresponding functional molecules in vivo that was not appreciated by in vitro analysis or by examination of structural orthologs. Mouse DAF is not expressed in early embryos and trophoblasts (*13*), and mouse MCP is exclusively expressed in the testes (*18*), thus leaving Crry as the critical regulator of complement activation during early murine embryonic development. These observations explain the difference in phenotype of the Crry-deficient embryos and mutant mice lacking DAF, in

which embryonic development is not affected (*19*). In contrast, DAF and MCP are heavily expressed in human placentas (*16*), and there is no direct human counterpart to Crry. Thus, human DAF or MCP should play a similar role as mouse Crry during early embryonic development by controlling effector components of natural immunity, in the form of complement regulation, to protect fetomaternal tissues from tissue inflammation and destruction.

**References and Notes**


1. W. O. Weigle, M. G. Goodman, E. L. Morgan, T. E. Hugli, *Springer Semin. Immunopathol.* **6**, 173 (1983); T. Kinoshita, *Immunol. Today* **21**, 291 (1991).
2. A. K. Abbas, A. H. Lichtman, J. S. Pober, *Cellular and Molecular Immunology* (Saunders, Philadelphia, PA, ed. 3, 1997), pp. 294–316.
3. V. M. Holers, T. Kinoshita, H. D. Molina, *Immunol. Today* **13**, 231 (1992); D. Hourcade, V. M. Holers, J. P. Atkinson, *Adv. Immunol.* **45**, 381 (1989); D. M. Lublin and J. P. Atkinson, *Curr. Top. Microbiol. Immunol.* **153**, 123 (1989).
4. C. B. Kurtz, M. S. Paul, M. Aegerter, J. J. Weis, J. H. Weis, *J. Immunol.* **143**, 2058 (1989); W. Wong and D. T. Fearon, *J. Immunol.* **134**, 4048 (1985).
5. H. Molina *et al.*, *J. Exp. Med.* **175**, 121 (1992).
6. Y.-U. Kim *et al.*, *J. Exp. Med.* **181**, 151 (1995).
7. B. W. Boom, A. M. Mommaas, M. R. Daha, B. J. Vermeer, *J. Dermatol. Sci.* **2**, 308 (1991); J. Itoh *et al.*, *Clin. Exp. Immunol.* **83**, 364 (1991).
8. Supplementary information is available at www.sciencemag.org/feature/data/1045819.shl.
9. The murine *Crry* gene was isolated from a sv129 cell genomic library by the use of Crry cDNA as a probe (*5*). A 6.7-kb genomic Eco RI DNA fragment containing exons 5 and 6 of the *Crry* gene was subcloned into pBluescript KS (Stratagene). The coding sequence was interrupted by replacing a portion of exon 5 with the 1.6-kb neomycin resistance cassette, pGKneobpA [H. Molina *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **93**, 3357 (1996)]. In this targeting vector, the pGKneobpA is flanked by a 1.7-kb genomic *Crry* fragment on the 5′ side and a 5-kb genomic *Crry* fragment on the 3′ side. Transfection of ES cells and blastocyst injection were done as described [B. Hogan, F. Costantini, E. Lacey, *Manipulating the Mouse Embryo: A Laboratory Manual* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1986), pp. 152–203].
10. DNA from individual ES cell clones or from tails of 3-week-old mice was isolated [P. W. Laird *et al.*, *Nucleic Acids Res.* **19**, 4293 (1991)]. Southern blotting was standard [J. Sambrook, E. F. Fritsch, T. Maniatis, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989), pp. 9.31–9.57]. Filters were hybridized overnight with a 0.5-kb probe derived from the *Crry* intronic area 5′ of the targeting construct sequence.
11. Single cell suspensions were prepared from primary fibroblasts derived from 13.5-dpc embryos. Crry staining was done with rabbit antibody to mouse Crry (1 μg) [B. Li *et al.*, *J. Immunol.* **151**, 4295 (1993)]. Binding was detected by the addition of fluorescein isothiocyanate–conjugated goat anti-rabbit antibody (Southern Biotechnology Associates, Birmingham, AL). Flow cytometry was done with a FACScan (Becton-Dickinson).
12. Deciduae were removed and frozen quickly in OCT compound (Miles, Elkhart, IN), and 10-μm-thick sections were cut. Endogenous peroxidase was quenched with 0.2% $H_2O_2$ in methanol. Sections were first incubated with rabbit antibody to mouse Crry followed by goat anti-rabbit immunoglobulin G (IgG) conjugated to horseradish peroxidase (HRP) (Southern Biotechnology) or with goat anti-mouse C3 (Cappel, Aurora, OH) followed by rabbit anti-goat IgG conjugated to HRP (Southern Biotechnology). Bound HRP was detected with diaminobenzidine. Sections were counterstained with 1% methyl green and covered with Crystal/Mount (Biomeda, Foster City, CA).
13. C. Xu *et al.*, unpublished data.
14. A. Circolo *et al.*, *Immunopharmacology* **42**, 135 (1999).
15. Genotype analysis of 41 littermates from $C3^{-/-}$ $Crry^{+/-}$ matings revealed that 13 (32%) were $Crry^{+/+}$, 17 (41%) were $Crry^{+/-}$, and 11 (27%) were $Crry^{-/-}$.
16. I. A. Rooney, T. J. Oglesby, J. P. Atkinson, *Immunol. Res.* **12**, 276 (1993); C. H. Holmes and K. L. Simpson, *Bailliere's Clin. Obstet. Gynaecol.* **6**, 439 (1992); C. H. Holmes *et al.*, *J. Immunol.* **144**, 3099 (1990); W. D. Billington, *Bailliere's Clin. Obstet. Gynaecol.* **6**, 417 (1992).
17. J. N. Bulmer, *Bailliere's Clin. Obstet. Gynaecol.* **6**, 461 (1992).
18. A. Tsujimura *et al.*, *Biochem. J.* **330**, 163 (1998).
19. X. Sun *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **96**, 628 (1999).


20. We thank H. Colten for $C3^{-/-}$ mice, P. Morgan for antibody to mouse DAF, and W. Yokoyama for critical comments. All experimental procedures and care of the animals were carried out in compliance with guidelines established by NIH and approved by the Division of Comparative Medicine at Washington University School of Medicine. Supported by grants from the National Institute of Allergy and Infectious Diseases (grants RO1 AI40576-01 and RO1 AI44912-01) to H.M. and by the Veteran's Administration Merit Award to H.M.

29 September 1999; accepted 1 December 1999


# Rad6-Dependent Ubiquitination of Histone H2B in Yeast


**Kenneth Robzyk, Judith Recht,* Mary Ann Osley†**



**Although ubiquitinated histones are present in substantial levels in vertebrate cells, the roles they play in specific biological processes and the cellular factors that regulate this modification are not well characterized. Ubiquitinated H2B (uH2B) has been identified in the yeast *Saccharomyces cerevisiae*, and mutation of the conserved ubiquitination site is shown to confer defects in mitotic cell growth and meiosis. uH2B was not detected in *rad6* mutants, which are defective for the ubiquitin-conjugating enzyme Ubc2, thus identifying Rad6 as the major cellular activity that ubiquitinates H2B in yeast.**


Modulation of chromatin structure by the posttranslational modification of histones has emerged as an important mechanism for regulating chromosome function in eukaryotes. Although acetylation of the histone $NH_2$-termini has been shown to be intimately connected to transcriptional regulation, the biological roles of other histone modifications remain obscure (*1*). A noteworthy modification is the conjugation of ubiquitin to the COOH-termini of the core histones H2A, H2B, and H3 (*2*). Ubiquitinated forms of these histones are stable in vivo, and their incorporation into nucleosomes has been proposed to alter chromatin structure locally (*2, 3*). Although the precise cellular roles of histone ubiquitination are unclear, this modification has been correlated with increased transcriptional activity, replication, and meiosis in higher eukaryotes (*3, 4*).

Ubiquitin is transferred to target proteins in a reaction catalyzed by members of a large group of ubiquitin-conjugating enzymes (Ubc's), which donate ubiquitin to the ε-amino group of specific lysine residues, often in a substrate-specific manner (*5*). Two evolutionarily conserved Ubc's, Rad6/Ubc2 and Cdc34/Ubc3, are able to ubiquitinate histones in vitro without the mediation of an E3 ubiquitin ligase (*6, 7*). However, neither protein has been demonstrated to ubiquitinate histones in vivo, and the Ubc that targets histones in cells remains to be identified. Here, we present evidence that histone H2B is ubiquitinated in yeast. We also show that attachment of ubiquitin to this core histone depends primarily on the activity of Rad6/Ubc2 and is required for both optimal mitotic cell growth and meiosis.

It has been reported that *Saccharomyces cerevisiae* contains little, if any, uH2A or uH2B (*8*). We reinvestigated this issue using combined genetic and immunological approaches. Lysine-to-arginine (K → R) substitutions were introduced at the conserved ubiquitination sites of both H2A and H2B (*9*). A single K → R substitution at $Lys^{123}$ in H2B (*htb1-K123R*) was combined with four K → R substitutions at $Lys^{119}$, $Lys^{120}$, $Lys^{123}$, and $Lys^{126}$ of H2A [*hta1-K119R, K120R, K123R, K126R* (hereafter *hta1-4K/R*)] to eliminate the possibility that, in the absence of the preferred H2A ubiquitination site ($Lys^{119}$), adjacent lysine residues ($Lys^{120}$, $Lys^{123}$, or $Lys^{126}$) could serve as acceptors for ubiquitin conjugation. Strains that contained the fully mutant forms of H2A plus H2B (*hta1-4K/R* + *htb1-K123R*) were viable (*9, 10*) but showed pronounced mitotic and meiotic defects. The mitotic phenotype was characterized by a small colony size on


Program in Molecular Biology, Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10021, USA.

*Present address: Department of Microbiology, Harvard Medical School, Boston, MA 02115, USA.
†To whom correspondence should be addressed. E-mail: m-osley@ski.mskcc.org