A Mutant *Drosophila* Insulin Receptor Homolog That Extends Life-Span and Impairs Neuroendocrine Function

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The *Drosophila melanogaster* gene *insulin-like receptor* (*InR*) is homologous to mammalian insulin receptors as well as to *Caenorhabditis elegans daf-2*, a signal transducer regulating worm dauer formation and adult longevity. We describe a heteroallelic, hypomorphic genotype of mutant *InR*, which yields dwarf females with up to an 85% extension of adult longevity and dwarf males with reduced late age-specific mortality. Treatment of the long-lived *InR* dwarfs with a juvenile hormone analog restores life expectancy toward that of wild-type controls. We conclude that juvenile hormone deficiency, which results from *InR* signal pathway mutation, is sufficient to extend life-span, and that in flies, insulin-like ligands nonautonomously mediate aging through retardation of growth or activation of specific endocrine tissue.

Molecular similarity between fly *InR* and worm *daf-2* suggests that mutants of *InR* in flies should affect adult life-span, as do mutants of *daf-2* in worms. *InR* and *daf-2* are members of the insulin receptor family with homology to mammalian insulin and insulin-like growth factor–1 (IGF-1) receptors (*1*, *2*). Worms carrying temperature-sensitive mutations in *daf-2* form dauers at high temperature, but at lower temperature develop directly into adults with extended longevity and resistance to exogenous stress (*3*, *4*). Genotypes homozygous for mutant *InR* have been reported to be lethal (*5*, *6*), but several heteroallelic combinations of *InR* alleles produce viable, dwarf adults that are slow to develop: InR^{EC34}/InR^{E19} and InR^{GC25}/InR^{E19} [reported in (*5*)], and InR^{E19}/InR^{p5545} (Table 1). In addition, InR^{E19}/InR^{E19} was found to be viable and dwarf once crossed into a new isogenic background (*7*). Dwarf females eclose with extremely immature ovaries, and the egg chambers of young adults remain previtellogenic (Fig. 1, A through C).

Measurement of INR kinase activity (*8*) indicated that the InR^{p5545} and InR^{E19} alleles both confer loss of INR function (Fig. 2). Basal activity of heterozygotes $+/InR^{E19}$ and $+/InR^{p5545}$ was 45% of that of the wild type. Insulin stimulation increased kinase activity of INR from +/+ and $+/InR^{E19}$ flies by 60%, but only by 26% from $+/InR^{p5545}$ flies ($P < 0.05$, for insulin stimulation of $+/InR^{E19}$ versus $+/InR^{p5545}$). Basal kinase activity of INR from InR^{E19}/InR^{E19} and from InR^{p5545}/InR^{E19} flies was 14 and 11% of that of the wild type, respectively; neither were stimulated by insulin. InR^{p5545} is a P-element insertion in exon-1 (*5*, *6*); the molecular lesion of InR^{E19} has yet to be identified, but it does not appear to occur in the known coding region of the gene (*9*).

Life tables (*10*) of *InR* mutant adults were compared to concurrent cohorts of a wild-type coisogenic strain (Fig. 3). Dwarfs of InR^{EC34}/InR^{E19} and InR^{GC25}/InR^{E19} are short-lived. Dwarf InR^{E19}/InR^{E19} and nondwarf $+/InR^{p5545}$ have moderately reduced survival; nondwarf $+/InR^{E19}$ individuals are normal. In contrast, females of InR^{p5545}/InR^{E19} are 85% longer lived than wild-type controls and overall present reduced age-specific mortality. As described in the accompanying paper (*11*), the life-span of female *D. melanogaster* is also extended by mutation of the insulin receptor substrate homolog *chico*. Survivorship among male *InR* genotypes fol-

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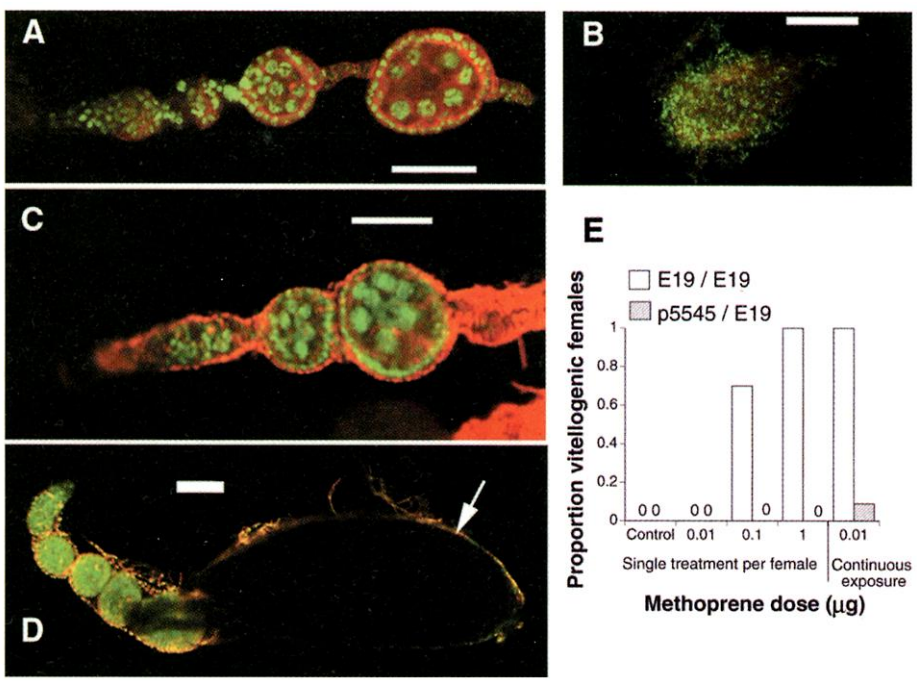

Fig. 1. Ovaries and ovarioles of wild-type and *InR* dwarf flies, stained with rhodamine-phalloidine and oligreen. Bars in (A) through (D), 50 μm. (**A**) Wild-type ovariole prepared 4 hours after eclosion. Germarium and stage 1-3 egg chambers are present. (**B**) InR^{E19}/InR^{E19} ovary 24 hours after eclosion. All ovarioles are immature. (**C**) InR^{E19}/InR^{E19} ovariole 11 days after eclosion. The degree of egg chamber maturation resembles that of newly eclosed wild-type flies [compare to (A)]. (**D**) A representative ovariole of dwarf InR^{E19}/InR^{E19} 11 days after a single topical application of methoprene (1 μg) at 1 day after eclosion. The gradient of maturation is typical for normal, virgin females: a mature stage 14 egg is silhouetted on the right (arrow), while previtellogenic stage 1-6 egg chambers are distal [stages described in (*35*)]. (**E**) Effect of methoprene treatment on vitellogenesis in *InR* mutant females. Single applications of methoprene in 0.1 μl acetone solvent were made upon the abdomen of anesthetized flies at 1 day after eclosion (control, acetone only). Females were dissected at 11 days after eclosion (10 to 22 flies per group) and scored as vitellogenic if any ovariole was at or beyond stage 8. For continuous exposure, methoprene was volatilized in glass culture bottles for 10 days (four applications of 0.01 μg methoprene per bottle).

lows the pattern observed for females. Relative to the wild type, InR^{p5545}/InR^{E19} males exhibit high mortality as early adults, but because of reduced mortality at late ages, dwarf life expectancy at 10 days is 43% greater than that of controls. It is likely that not all *InR* alleles increase longevity because the gene is highly pleiotropic, with some alleles producing developmental defects that carry over to the adult stage, which counterbalance positive effects of the allele upon aging.

The fact that *InR* mutants are nonvitellogenic suggests a plausible mechanism for the extended longevity of InR^{p5545}/InR^{E19} flies. *Drosophila* overwinter as adults in a reproductive diapause where egg development is arrested at previtellogenic stages (*12, 13*). In many insects, including *Drosophila*, reproductive diapause is proximally controlled through down-regulation of juvenile hormone (JH) synthesis by the corpora allata (CA) (*14, 15*). Ovaries of *InR* dwarf females morphologically resemble ovaries of diapause wild-type flies, and exogenous application of the JH analog methoprene to dwarf females initiated vitellogenesis (Fig. 1, C and D). Females of InR^{E19}/InR^{E19} responded to a single treatment of methoprene in a dose-dependent manner, but females of InR^{p5545}/InR^{E19} required continuous exposure to hormone to induce any vitellogenesis (Fig. 1E). Direct assay of adult JH synthesis (*16*) verified that CA activity was reduced in *InR* dwarfs to about 23% of the wild-type level (Fig. 4). Because reduced JH synthesis is seen in

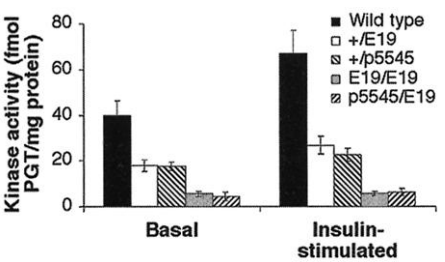

Fig. 2. Kinase activity of INR from membranes prepared from adult heads of defined genotypes. Membranes were solubilized and INR autophosphorylated in the absence or presence of insulin (1 μM), and immunoprecipitated with Ab dp1040. Each mean ± SEM represents data from three to six independent experiments for each genotype, each performed in triplicate.

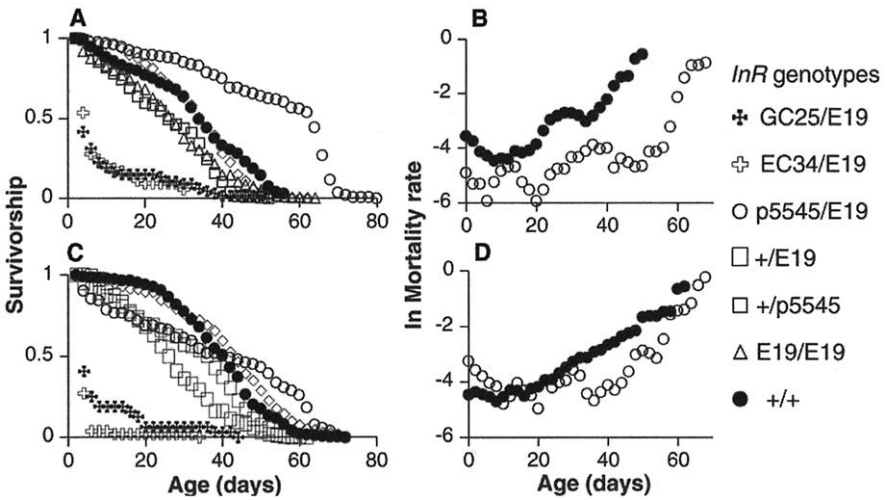

Fig. 3. Survivorship and age-specific mortality of *InR* mutant genotypes relative to the wild-type coisogenic control. Survivorship calculated as N_x/N_0, where N_x is the number alive beginning each census interval and N_0 is the initial cohort size. Age-specific mortality is smoothed first with a 3-day running average and presented as logarithm of hazard, $\ln(\mu_x) \approx \ln\{-[\ln(1-d_x/N_x)]\}$, where d_x is the number of deaths. Pairwise comparisons among genotypes for age-specific survival $(1-\mu_x)$ by log-rank test with significance corrected for multiple tests. (**A** and **B**) Among females, relative to the wild type, the age-specific survival of InR^{EC34}/InR^{E19}, InR^{GC25}/InR^{E19}, InR^{E19}/InR^{E19}, and $+/InR^{p5545}$ is reduced (smallest $\chi^2 = 5.22$, $P < 0.02$), survival of $+/InR^{E19}$ does not differ ($\chi^2 = 0.79$, $P = 0.37$), and survival of InR^{p5545}/InR^{E19} is increased ($\chi^2 = 175.1$, $P < 0.0001$). (**C** and **D**) Among males, relative to the wild type, age-specific survival of InR^{EC34}/InR^{E19}, InR^{GC25}/InR^{E19}, $+/InR^{p5545}$, and InR^{E19}/InR^{E19} is reduced (smallest $\chi^2 = 29.8$, $P < 0.001$), survival of $+/InR^{E19}$ does not differ ($\chi^2 = 4.89$, $P = 0.03$, nonsignificant under multiple tests), and survival of InR^{p5545}/InR^{E19} is increased ($\chi^2 = 21.4$, $P < 0.0001$).

Table 1. Phenotypes of *InR* dwarf and nondwarf genotypes relative to the coisogeneic wild-type control (mean ± SEM).

Genotype at *InR*	Development rate (days)*	Adult size (10^{-3} g)*	Fertility‡	Life expectancy at eclosion [days (SEM)]	SOD activity V/v (10^{-2})§	Triglycerides (μg/μg protein)‖	Volume O_2 consumed¶
				Dwarf			
InR^{E19}/InR^{E19}	19.6	0.43	F: sterile	F: 24.6 (0.98)	1.3	4.48	0.079
	(0.38)	(0.01)	M: 20%	M: 15.27 (1.1)	(0.6)	(1.15)	(0.013)
InR^{E19}/InR^{p5545}	21.6	0.40	F: sterile	F: 60.1 (0.9)	2.1	4.81	0.060
	(0.53)	(0.01)	M: 35%	M: 39.0 (5.1)	(0.6)	(1.0)	(0.002)
InR^{EC34}/InR^{E19}	21†	48%†	F: sterile †	F: 2.3 (0.53) M: 1.4 (0.19)			
InR^{GC25}/InR^{E19}	19†	56%†	F: sterile †	F: 1.7 (0.21) M: 1.7 (0.29)			
				Nondwarf			
InR^{p5545}/InR^{+}	12.4	1.01		F: 23.7 (1.2)	0.53	0.73	
	(0.18)	(0.02)		M: 30.5 (0.16)	(0.04)	(0.07)	
InR^{E19}/InR^{+}	11.8	1.02		F: 32.1 (0.96)	0.51	0.52	
	(0.16)	(0.02)		M: 39.4 (1.1)	(0.1)	(0.90)	
InR^{+}/InR^{+}	11.9	1.01		F: 32.3 (1.1)	0.87	0.90	0.065
	(0.13)	(0.02)		M: 35.5 (1.3)	(0.2)	(0.19)	(0.010)

*Development rate and adult body size were measured from the eclosion time and size of the first 10 adults of each sex; eggs were laid at constant density (50/vial). †Reported in (*5*). ‡Male fertility estimated as proportion of fertilized eggs produced by 20 males individually paired with wild-type females, and scaled to the fertility of wild-type males. §Enzyme activity measured by inhibition rate of substrate reduction (v) normalized by substrate reduction when Cu/Zn–superoxide dismutase (SOD) was inactivated (V); scaled by total protein. Estimates measured from five samples each of three females at 3 days old. ‖Triglycerides per adult estimated from five samples each of three females at 3 days old. ¶ Volume of oxygen consumed (μl/mg/hour) measured from 100 flies (1:1 sex ratio) at 25°C over 4 hours in a closed 60-ml chamber. Values are the mean of four replicate measures per genotype made for cohorts between 2 to 8 days in age.

InR^{E19}/InR^{E19} flies, which exhibit normal life-span, as well as in long-lived InR^{p5545}/InR^{E19} flies, the simple lack of JH may not be enough to extend longevity.

Loss of corpora allata JH accounts for dwarf infertility. Mutation of *InR* may increase longevity because infertility reduces allocation of metabolic resources to reproduction and frees resources for somatic maintenance (*17*) or because reduced JH in mutant flies induces specific physiological mechanisms of somatic persistence normally expressed during adult reproductive diapause. Adult *D. melanogaster* in reproductive diapause age at negligible rates and are stress resistant; these traits are reversed by treatment with methoprene (*18*). Extended survival is characteristic of adult reproductive diapause in acridid grasshoppers and in the monarch butterfly, and surgical ablation of the corpora allata to eliminate adult JH synthesis induces both diapause and increased longevity [reviewed in (*19*)]. Consistent with the notion that reduced JH synthesis can directly extend life-span, *InR* dwarf flies show somatic physiological changes (*20*) (Table 1): (i) triglycerides are elevated fourfold ($F = 32.2$, $P < 0.001$), as observed in diapause *D. triauraria* (*21*) and in dwarf *D. melanogaster* mutant for *chico* (*22*), and (ii) Cu/Zn-superoxide dismutase concentration is increased twofold ($F = 9.42$, $P < 0.02$), as is characteristic of long-lived mutants of *Caenorhabditis elegans* (*23*). Measured in young adults, no difference in mass-specific metabolic rate was detected (Table 1). We suggest that infertility need not be the direct cause of slowed aging in *InR* mutants; JH may simply control both fertility and life-span.

To test directly whether JH modulates survival in InR^{p5545}/InR^{E19} female dwarfs, we investigated whether treatment with methoprene restores wild-type longevity to these mutants, even if it does not fully restore fertility (*24*). In concurrent trials of dwarf and wild-type flies, survival of methoprene-treated InR^{p5545}/InR^{E19} females was reduced toward the level observed in coisogenic controls (Fig. 5). This rescue is physiological rather than toxicological because, in wild-type controls, methoprene produced no significant change relative to ethanol-treated flies.

The *InR* pathway may alter endocrine function in two ways. Adult CA is derived from neurosecretory tissue of the larval ring gland. Adult dwarf CA may be immature upon metamorphosis as a result of cell autonomous effects of *InR* upon the development of neuroendocrine cells. A second way *InR* may alter endocrine function is that JH secretion by CA may be impaired by reduced neuropeptide transmission in the adult brain (*25*), due to a reduction of INR function in brain areas where it is normally expressed (*26*).

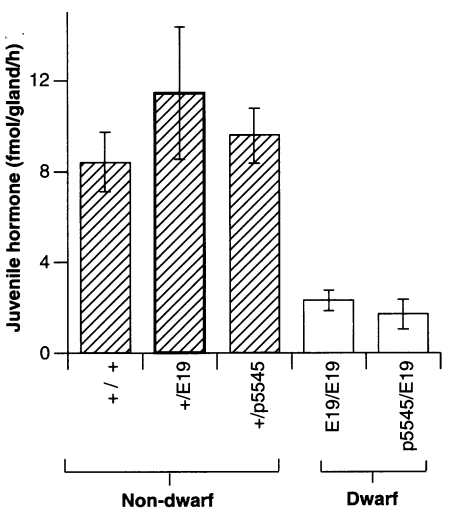

Fig. 4. Synthesis rates of juvenile hormone (JH) by incubated corpora allata isolated from 2-day-old wild-type and *InR* mutant females (mean ± SEM, replicate assays). JH is calculated from sum of counts across fractions identified as JHB3, JHIII, and methyl farsinate. Significant differences occur only between dwarfs and nondwarfs [$F(4,14) = 6.57$, $P < 0.005$].

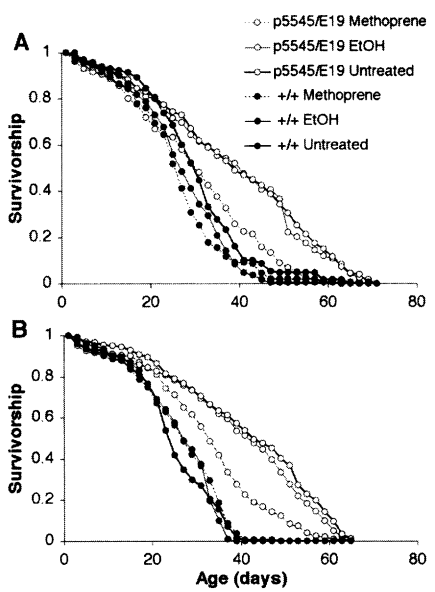

Fig. 5. Survival of female dwarf and coisogenic wild-type cohorts treated with methoprene in ethanol, ethanol (EtOH) only, or no solution. Methoprene per application: **(A)** 0.02 μg, **(B)** 0.002 μg. Mortality of methoprene-treated InR^{p5545}/InR^{E19} is at least 90% greater [in (A)] and 72% greater [in (B)] than either ethanol treatment alone or untreated dwarfs [Cox-proportional hazard, (A) and (B), respectively: exp(coef.) = 1.898, $P < 0.0001$; exp(coef.) = 1.721, $P < 0.0001$]. Mortality of methoprene-treated wild type does not differ significantly in (A) from its ethanol-treated control ($\chi^2 = 0.34$, $P = 0.56$), or in (B) from its ethanol-treated or untreated controls ($\chi^2 = 2.16$, $P = 0.34$).

In *C. elegans*, the insulin/IGF-1 pathway influences dauer formation, fertility, and aging in part through nonautonomous, secondary signaling; sterility is not required for extended longevity in *C. elegans* because some long-lived *daf-2* are fully fertile (*27*). For *Drosophila*, we have shown that *InR* affects neurosecretory tissue specialized for secretion of juvenile hormone. Therefore, mutations in the insulin signaling pathway in flies autonomously affect cell proliferation, growth, and body size (*5, 22*), but nonautonomously affect diapause, reproduction, and life-span through effects upon specific neuroendocrine cells. Deficiency in a juvenoid-like hormone signal in worms and in flies may extend longevity because its absence leads to the inappropriate expression of parallel physiological programs normally reserved for dauer or diapause.

This invertebrate model may have parallels with mammalian aging. Ames and Snell mice are mutant for the genes *Prop-1* or *Pit-1*, respectively, and are defective for pituitary development [reviewed in (*28*)]. Consequently, they are deficient in growth hormone, prolactin, and thyroid-stimulating hormone, leading to hypothyroidism and presumably reduced synthesis of thyroxin, a retinoid hormone with potential functional similarity to JH (*29*). These mice are phenotypically dwarf, mildly obese, and long-lived (*28*). A remarkably similar phenotype is observed in mice lacking insulin receptor function in the central nervous system or those lacking the *chico* homolog, IRS-2, in all tissues: increased fat mass and infertility with accompanying neuroendocrine deficiency (*30, 31*). Although effects on life-span in these mice remain to be determined, the concordance of phenotypes suggests that insulin signaling may be central to a common mechanism that exists across taxa for the neuroendocrine regulation of metabolism and the reproductive state, and their associated consequences upon aging.

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7. Balancer stocks of *InR* (*p5545*, *GC25*, *EC34*, *E19*) were provided by J. Jack (University of Connecticut Medical Center); a third-chromosome isogenic strain marked with *radius incompletus* (*ri*), *red eye* (*r*), and *ebony* (*e*) was provided by K. Wharton (Brown University); and InR^+ in a wild-type background occurring in an isofemale line from Windsor, Canada, was provided by M. B. Sokolowski (University of Toronto). Each *InR* mutant allele was backcrossed to the *ri r e* strain for 10 generations by selection for e^+, and

progeny tested at generation 10. To construct the wild-type coisogenic strain, we backcrossed Windsor into *ri r e* for 10 generations by selecting e^+ daughters to yield *ri r InR*$^+$ e^+ / *ri r InR*$^+$ e^+.
8. Membranes were prepared from adult heads and immunoprecipitated with an INR-specific polyclonal antibody. Kinase activity was measured as described (*32*).
9. S. Sciacchitano *et al.*, unpublished data.
10. Life tables were constructed at 25°C by the extinct cohort method. Within a 24-hour eclosion period, 100 adults of mixed sex were introduced into 1-liter demography cages. Every 2 days, dead individuals were aspirated from cages and counted, and fly-medium vials were changed. Three to four replicate cages were concurrently assayed for each genotype.
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REPORTS

Role of Histone H3 Lysine 9 Methylation in Epigenetic Control of Heterochromatin Assembly

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The assembly of higher order chromatin structures has been linked to the covalent modifications of histone tails. We provide in vivo evidence that lysine 9 of histone H3 (H3 Lys^9) is preferentially methylated by the Clr4 protein at heterochromatin-associated regions in fission yeast. Both the conserved chromo- and SET domains of Clr4 are required for H3 Lys^9 methylation in vivo. Localization of Swi6, a homolog of *Drosophila* HP1, to heterochomatic regions is dependent on H3 Lys^9 methylation. Moreover, an H3-specific deacetylase Clr3 and a β-propeller domain protein Rik1 are required for H3 Lys^9 methylation by Clr4 and Swi6 localization. These data define a conserved pathway wherein sequential histone modifications establish a "histone code" essential for the epigenetic inheritance of heterochromatin assembly.

The organization of the higher order chromatin structure has been linked to the posttranslational modifications of histone tails, including acetylation, phosphorylation, and methylation (*1*). It has been suggested that distinct combinations of covalent histone modifications, also referred to as the "histone code," provide a "mark" on the histone tails to recruit downstream chromatin-modifying proteins (*2*, *3*). This is best illustrated by recent studies indicating that the conserved bromodomain of several transcriptional coactivators bind specifically to acetylated lysine residues on histone tails (*4*, *5*). The mechanisms responsible for the establishment and maintenance of multiple covalent modifications within the same or different histone tail are not fully understood.

Modifications of histone tails have also been linked to heterochromatin assembly. Histones H3 and H4 are largely hypoacetylated in heterochromatic chromosomal regions in organisms as diverse as yeast, flies, and mammals (*6*–*8*). In fission yeast, hypoacetylation of histones is associated with the silent mating-type region and centromeres (*9*, *10*), chromosomal domains that share many parallels with heterochromatic regions in higher eukaryotes (*11*). Centromeric regions comprising a central core of unique sequences surrounded by inner (*imr*) and outer (*otr*) repeats are assembled into silenced chromatin structures (*12*). Similarly, a large ~15-kb chromosomal domain at the mating-type (*mat2/3*) region, including the *mat2* and *mat3* loci and an interval between them, known as the *K*-region, is maintained in a silent epigenetic state (*13*, *14*). Among the trans-acting factors that affect silencing at these regions, Clr3 and Clr6 belong to family of histone deacetylases (HDACs) (*15*). Swi6 and Clr4 proteins contain a chromodomain, an evolutionarily conserved motif initially identified in HP1 and *Polycomb* proteins (*16*–*18*). Recently, both Clr4 and its mammalian counterpart, SUV39H1, have been shown to have intrinsic histone H3-specific methyltransferase (HMTase) activity in vitro (*19*). However, it is not known whether histones are the physiological targets of these methyltransferases in vivo.

Consistent with previous findings, recombinant Clr4 (rClr4) was found to contain HMTase activity exclusively for histone H3 (Fig. 1B). To identify the specific residue of H3 methylated by rClr4, we used synthetic peptides derived from the NH_2-terminus of H3 as substrates in an in vitro HMTase assay (*20*, *21*). Clr4 preferentially methylated the H3 1-20 unmodified peptide but failed to methylate the H3 19-35 unmodified peptide (Fig. 1C), indicating that the target residue of Clr4 HMTase resides in the first 20 amino acids of H3. To determine this target residue, we developed a synthetic H3 1-20 peptide set that contained covalent modifications on different amino acids. With these peptides as substrates, only acetyl or methyl modifications on Lys^9 effectively blocked rClr4 HMTase activity, indicating that Clr4, like its mammalian homolog SUV39H1 (*19*), selectively methylates Lys^9 of H3. Furthermore, similar to SUV39H1, rClr4 HMTase activity

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