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Methylation at lysine 4 of histone H3 in ecdysone-dependent development of *Drosophila*

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Abstract

Steroid hormones fulfil important functions in animal development. In *Drosophila*, ecdysone triggers moulting and metamorphosis through its effects on gene expression¹. Ecdysone works by binding to a nuclear receptor, EcR, which heterodimerizes with the retinoid X receptor homologue Ultraspiracle^{2,3}. Both partners are required for binding to ligand or DNA^{4–6}. Like most DNA-binding transcription factors, nuclear receptors activate or repress gene expression by recruiting co-regulators, some of which function as chromatin-modifying complexes^{7,8}. For example, p160 class coactivators associate with histone acetyltransferases and arginine histone methyltransferases⁹. The *Trithorax-related* gene of *Drosophila* encodes the SET domain protein TRR. Here we report that TRR is a histone methyltransferase capable of trimethylating lysine 4 of histone H3 (H3-K4). *trr* acts upstream of *hedgehog* (*hh*) in progression of the morphogenetic furrow, and is required for retinal differentiation. Mutations in *trr* interact in eye development with *EcR*, and EcR and TRR can be co-immunoprecipitated on ecdysone treatment. TRR, EcR and trimethylated H3-K4 are detected at the ecdysone-inducible promoters of *hh* and *BR-C* in cultured cells, and H3-K4 trimethylation at these promoters is decreased in embryos lacking a functional copy of *trr*. We propose that TRR functions as a coactivator of EcR by altering the chromatin structure at ecdysone-responsive promoters.

TRR¹⁰ was identified as a protein that is closely related to the well-known *HOX* gene activator Trithorax (TRX). An antibody to TRR recognized a protein band of the expected size (relative molecular mass (M_r) 260) in nuclear extracts from embryos (Fig. 1b), suggesting that TRR is a nuclear protein. Nuclear localization of TRR was confirmed by immunohistochemical staining of salivary glands from third instar larvae (Fig. 1c). TRR contains several protein domains that are conserved among transcriptional regulators (Fig. 1a), including a SET domain. This ancient domain is known to bind to several proteins, including histones¹¹, and to have histone methyltransferase (HMTase) activity (reviewed in ref. ¹²). Indeed, TRR from nuclear extracts bound to a column containing the four core histones; and the isolated SET domain of TRR, like the closely related TRX SET¹¹, bound to histones H3 and H4 (Supplementary Fig. 1a, b).

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Purified TRR SET showed histone-H3-specific HMTase activity *in vitro* (Fig. 1d, e). It also methylated a histone H3 amino-terminal peptide that was either unmodified or trimethylated at lysine 9 (K9), but it did not methylate a similar peptide that was already trimethylated at K4 (Fig. 1d). To test whether other lysine residues known to be methylated in histone H3 are methylated by TRR SET, we tested histones mutated at K4, K27, K36 and K79 (ref. ¹³). Only mutation of K4 affected the ability of histone H3 to be methylated (Fig. 1e). K4 specificity was confirmed by a failure of antibodies specific for H3-MeK27 and H3-MeK36 to recognize the product of TRR methylation (data not shown). K4 was directly confirmed as a target of TRR methylation in histone H3 by Edman degradation of H3 methylated *in vitro* by TRR SET (Fig. 1f). Further analysis showed that TRR SET can trimethylate at H3-K4 (Fig. 1g). Because trimethylated H3-K4 is associated with high transcriptional activity¹⁴, these results indicate that TRR may function as a transcriptional coactivator.

Ash1 and TRX are also H3-K4 HMTases in *Drosophila*^{15,16}. TRR seemed to be more abundant in embryos than either Ash1 or TRX (data not shown), and it was associated with many more sites on salivary gland polytene chromosomes (Fig. 2c). To test the magnitude of TRR's contribution to H3-K4 methylation, we compared the amounts of H3-MeK4 in extracts from wild-type embryos with those from embryos homozygous mutant for the *trr*¹ null allele¹⁰. Mutation of *trr* markedly reduced the quantity of both di- and trimethylated H3-K4 (Fig. 1h). By contrast, we detected only a small decrease in methylated H3-K4 in embryos homozygous for a *trx* null mutation (*trx*^{B11}, data not shown). We conclude that TRR is a major H3-K4 HMTase in *Drosophila*.

Unlike TRX, TRR contains four sequences that match the nuclear-receptor-interacting motifs¹⁷ LXXLL or LLXXL ('NR' in Fig. 1a). We tested each TRR NR region for its ability to interact with the ecdysone receptor *in vitro*. The NR2 domain of TRR, which contains an LXXLL motif, bound to full-length EcR and Ultra-spiracle (USP) proteins; although EcR and USP are considered to be obligate heterodimer partners, either protein bound to the TRR NR2 domain alone (Fig. 2a). The three other NR domains of TRR showed very little if any interaction with EcR and USP (data not shown). An association between TRR and the ecdysone receptor *in vivo* was shown by co-immunoprecipitation from embryo extracts (Fig. 2b), in which TRR was associated with both components of the ecdysone receptor.

We confirmed the association of TRR and EcR *in vivo* by two additional experiments. Like TRX¹⁸, TRR bound to several sites on salivary gland polytene chromosomes; immunostaining of polytene chromosomes showed an almost complete colocalization of TRR with EcR-B1 (Fig. 2c), the predominant EcR isoform of the salivary gland. Although the stained bands were numerous, they constituted only a few of the bands visible by DNA staining (blue). By contrast, there was little overlap between the staining for TRR and either TRX or Polycomb (data not shown). In the eye imaginal disc, TRR was expressed in a pattern essentially identical to those of USP and EcR-A, the predominant EcR isoform of eye discs (Fig. 2d). As reported for USP¹⁹, both TRR and EcR-A were expressed primarily in the region posterior to the morphogenetic furrow and in smaller amounts in a row of cells just anterior to the morphogenetic furrow, and all three proteins were absent from most of the region anterior to the morphogenetic furrow. Thus, in both the salivary gland and the eye disc, there is marked spatial colocalization of TRR and EcR-USP.

Having established a physical association between TRR and the ecdysone receptor, we tested for a functional association *in vivo*, concentrating on the developing compound eye. Ecdysone and its receptor have complex roles in both progression of the morphogenetic furrow^{19,20} and post-furrow differentiation²¹. We showed that TRR is required for eye development by examining the phenotype of somatic *trr*¹ clones in the adult eye. Two abnormalities were visible in the clonal patches: a notching of the anterior boundary of the eye, interpretable as a

retardation of morphogenetic furrow progression; and a ‘rough eye’ phenotype, attributable to defects in photoreceptor differentiation (Fig. 2e).

Three approaches showed that *trr* interacts genetically with genes encoding components of the ecdysone receptor and the ecdysone synthesis pathway. First, heterozygosity for the null allele *trr*¹, similar to the temperature-sensitive *ecdysoneless* allele *ecd*¹ (at its partially restrictive temperature, 25 °C), enhanced the furrow-stop phenotype of the *B*¹ mutation (Supplementary Fig. 2a, b). Introducing an extra copy of *trr*⁺ as a transgene¹⁰ reversed the *trr*¹ effect (Supplementary Fig. 2a). Flies with a combination of *ecd*¹, *trr*¹ and *B*¹ showed an additional defect in morphogenetic furrow progression, particularly in the ventral part of the eye (Supplementary Fig. 2b, bracket). *trr*³, in which the SET domain is deleted, was similar to *trr*¹ in its interaction with *B*¹ (data not shown). These genetic interactions are consistent with a role for TRR as a coactivator for the ecdysone receptor.

Second, expression of the dominant-negative mutant EcR-F645A in the post-morphogenetic furrow retinal epithelium by the *GMR-GAL4* driver led to severe defects in eye differentiation (Fig. 2f and Supplementary Fig. 2d) and in a marked lethality before adult eclosion (ref. ¹⁰ and Table 1). Under the conditions used, lethality was apparently due largely to desiccation, presumably caused by defective cuticle secretion. The lethality associated with EcR-F645A was strongly enhanced in *trr*¹ and *trr*³ heterozygotes (Table 1). Inclusion of an extra copy of *trr*⁺ partially suppressed both the lethality and the eye phenotype (Table 1 and Fig. 2f). A mutation in *Smrter* (*Smr*), which encodes a known co-repressor for the ecdysone receptor²², showed an interaction with EcR-F645A similar to that observed with an extra copy of *trr*⁺ (Table 1 and Fig. 2f). Thus, *trr* and *Smr* function antagonistically, which is again consistent with a role for TRR as a coactivator for the ecdysone receptor.

Third, heterozygosity for the EcR null allele *EcR*^{M554fs} noticeably suppressed the furrow-stop phenotype of *trr*¹/*B*¹ flies (Fig. 2g, arrows). Homozygotes for the viable allele *trr*⁴, like the *trr*¹ null clones, showed both defects in morphogenetic furrow progression and disorganization of the ommatidial array (Fig. 2h; *trr*⁴ did not complement *trr*¹ (data not shown) and is associated with insertion of a P-element in the *trr* promoter region). Both *trr*⁴ phenotypes were suppressed by heterozygosity for *EcR*^{k06210}, an isoform-A-specific hypomorphic mutation, which by itself caused no visible eye phenotype (Fig. 2h, left).

In contrast to the results from the first two approaches, these interactions between *trr* and a reduction in EcR function indicate that TRR may function in an opposite direction to EcR. These apparently paradoxical results are readily explained by reference to the fact that ecdysone converts its receptor from a transcriptional repressor to an activator. Both *ecd*¹ and EcR-F645A specifically abolish the activation function without altering repression²³, and both enhance the phenotype of *trr* mutations, consistent with a coactivator function for TRR. Mutations in EcR affect both receptor functions and suppress the phenotype of *trr* mutations. This suggests that EcR-mediated repression has an essential, dose-sensitive role that can be partially overcome by a decrease in coactivator (TRR) titre. Similarly complex effects in eye development have been seen for mutations in *usp*²⁴.

We sought to clarify the role of TRR in furrow progression. Our analyses of genetic interactions between *trr* and both *hedgehog* (*hh*) and its downstream target *decapentaplegic* (*dpp*) indicated that TRR has a role in Hedgehog (Hh) signalling (Fig. 3). Like *B*¹, *trr*¹ was found to be a dominant enhancer of the eye-specific mutation *dpp*^{blk} (ref. ²⁵ and Fig. 3a). Hh is involved in morphogenetic furrow progression and in post-furrow photoreceptor differentiation²⁶, and *trr* mutations altered *hh* phenotypes in both processes. Although a reduction in *trr* dose had no apparent effect on either aspect of the *hh*¹ phenotype (data not shown), an extra copy of *trr* visibly suppressed the furrow-stop phenotype and restored normal ommatidial differentiation

in *hh^l* homozygotes. Flies heterozygous for both *trr^l* and *hh^{l3C}* also showed a rough-eye phenotype that was not produced by either mutation alone (Fig. 3b).

Because heterozygosity for *trr^l* enhanced the morphogenetic furrow progression defect of *B^l* flies (Supplementary Fig. 2a), we tested whether it also enhanced the reduction in *dpp* and *hh* expression caused by *B^l*. Heterozygosity for *trr^l* alone had no effect on eye morphology or on *hh* and *dpp* expression (data not shown). *B^l* caused a decrease in expression of *dpp* in the middle of the eye disc, whereas *dpp* expression was completely abolished in this region in *B^l/trr^l* transheterozygotes (Fig. 3c). *trr^l* also clearly enhanced the deficit in *hh* expression caused by *B^l* (Fig. 3d). The requirement of *trr* for *hh* expression was further shown by the considerable decrease in *hh* expression in the *trr* null clones posterior to the morphogenetic furrow (Fig. 3e). The spatial pattern of *trr* expression is consistent with TRR having a direct role in *hh* expression (Figs 2d and 3f), whereas there is little if any overlap between the areas of expression of *trr* and *dpp* (Fig. 3f). Because *hh* functions upstream of *dpp* in directing morphogenetic furrow progression, these results indicate that TRR may positively regulate *hh* transcription, and that a decrease in *hh* expression in *trr* mutants may lead to a decrease in the expression of *dpp*.

Alternatively, TRR might upregulate *dpp* directly, in addition to its indirect effect through *hh*. These possibilities can be distinguished by use of the dominant hypermorphic mutation *ro^{Dom}*: Hh stimulates *ro* expression, and *ro^{Dom}* interferes with *hh* induction of *dpp*^{27–29}. If *trr* directly upregulates *dpp*, then an extra copy of *trr* would be expected to suppress the *ro^{Dom}* phenotype. If the effect of TRR on *dpp* expression is mediated completely by *hh*, then an extra copy of *trr* would be expected to enhance the *ro^{Dom}* phenotype. We found that the latter is the case (Supplementary Fig. 2b). Therefore, the effects of TRR on *dpp* expression are likely to be predominantly a secondary consequence of its effects on *hh* expression.

The above experiments lead to the hypothesis that TRR functions as a coactivator for the ecdysone receptor, mediating ecdysone activation of transcription by modifying chromatin structure at target promoters. The results also indicate that *hh* may be a direct TRR target during eye development. This hypothesis predicts an ecdysone-dependent association of TRR with the ecdysone receptor and with ecdysone-responsive promoters, and an ecdysone induction of H3-K4 trimethylation at ecdysone-induced promoters. We tested these predictions as follows.

Fractionation of an embryo extract showed that TRR was present in very large complexes, $M_r \approx 2,000,000$ (2,000K), whereas EcR migrated as a broad band, predominantly at a much lower M_r . Treatment of the extract with ecdysone caused the TRR peak to become confined to a higher M_r range and caused a marked shift in migration of EcR to higher M_r , so that roughly half of the EcR co-migrated with TRR (Fig. 4a). Similar results were obtained with S2 cell extracts (data not shown). Antibody specific for EcR could precipitate TRR more efficiently from the 2,000K fraction of the hormone-treated embryonic extract than from the untreated extract (Fig. 4b). Thus, TRR and EcR are components of a hormone-dependent complex *in vivo*. Although the presence of USP in this complex has not been shown, the hormone dependence of the EcR–TRR interaction indicates that TRR may have a direct role in mediating transcriptional induction by the ecdysone receptor.

We then used S2 cells to examine the chromatin structure of two putative target promoters. We examined *hh*, which was suggested by our results to be a target of TRR activation in the eye disc, and *BR-C*, a gene whose transcription is ecdysone-responsive in many tissues, including the eye^{20,24}. Transcription of both genes was induced by ecdysone in S2 cells (Fig. 4c). Chromatin immunoprecipitation (ChIP) assays showed that ecdysone treatment caused a marked increase in the association of both promoters with EcR, TRR and H3 trimethylated at K4 (Fig. 4d). A causal connection between the presence of TRR and histone methylation at

these promoters was indicated by similar assays comparing extracts from wild-type embryos and from embryos homozygous for strong mutations at *trr*: transcription and the amount of H3-K4 trimethylation at both promoters was significantly reduced in embryos homozygous for either *trr*¹ or *trr*³ (Fig. 4e, f). In addition, because the SET domain of TRR is deleted in *trr*³ (Fig. 1a), this indicates that TRR SET may be responsible for the ecdysone-induced H3-K4 methylation at these promoters.

Our experiments show that TRR is a major *Drosophila* HMTase that binds to EcR in an ecdysone-dependent manner and causes H3-K4 trimethylation at ecdysone-inducible promoters. In the developing compound eye, TRR has a crucial role in progression of the morphogenetic furrow and in post-furrow differentiation of the retinal epithelium, and one of its direct targets is the *hh* promoter. Taken together, our results imply that TRR is a coactivator for the ecdysone receptor.

Methods

Fly strains, genetic and clonal analyses

Details of the genetic manipulations are given in the Supplementary Information.

Histology and immunocytochemistry

The anti-TRR polyclonal antiserum T1 was raised in rabbits (Cocalico Biological) against a bacterially expressed 6 × His fusion protein containing TRR residues 568–828. The T1 antiserum was affinity purified, and its specificity was confirmed by the absence of staining in homozygous *trr*¹ null mutant embryos (data not shown). Monoclonal antibodies to EcR-A (15G1a) and EcR-B1 (AD4.4) were a gift from C. Thummel. Immunostaining of polytene chromosomes and eye discs is described in the Supplementary Information.

Protein analysis

Western blotting, co-immunoprecipitation, *in vitro* binding assays and size fractionation of nuclear extract were done by standard procedures (see Supplementary Information) with the following reagents: antibodies against histone H3 either dimethylated at K4 (Upstate Biotechnology) or trimethylated at K4 (Abcam); T1 antibodies against TRR; antibodies against glutathione *S*-transferase (GST; diluted 1:1,000; Amersham Pharmacia Biotech); DDA2.7 monoclonal antibodies against the EcR common region (1:1,000; a gift from C. Thummel); and monoclonal antibodies against USP (1:50; a gift from F. Kafatos).

Semi-quantitative RT-PCR

Schneider S2 cells were grown in the presence of 10⁻⁶ M 20-hydroxy-ecdysone (20HE) for 24 h. RNA was extracted from roughly 10⁶ S2 cells or 50 embryos with a High Pure RNA Isolation kit (Roche), and reverse transcription was done with random hexamers and AMV reverse transcriptase. To normalize the relative amounts of *hh* and *BR-C Zl* RNAs, we first analysed samples by PCR with *rp49*-specific primers. Semi-quantitative PCR was achieved by preparing serial dilutions of input DNA. All samples were assayed in the range where the PCR products showed a linear correlation with input DNA. We used the following primers. For *rp49*: sense, 5'-cgg atc gat atg cta agc tg-3'; antisense, 5'-gaa cgc agg cga ccg ttg ggg-3'. For *hh*: sense, 5'-tga tcc caa cga tcc tgt-3'; antisense, 5'-gta aca gcg tct gtg ttc t-3'. For *BR-C Zl*: sense, 5'-ccg acc aat tgc aaa gat ct-3'; antisense, 5'-gta cat gga tct cag ctg ca-3'.

ChIP assays

Experiments were done by standard protocols and a ChIP Assay kit (Upstate Biotechnology), using the same starting material as in the RT-PCR experiments. For normalization, the

unprecipitated material was analysed by PCR using the primer set specific to the *hh* promoter. We used the following primers. For the *hh* promoter (−564 to −173): sense, 5'-tgg atg aaa gtg tgt gcc t-3'; antisense, 5'-acc act acc tga ggg gca tat-3'. For the *BR-C ZI* promoter (−554 to −120): sense, 5'-tcc att tgc gat gcg ttg gct-3'; antisense, 5'-agg ttc act gca gtt cag agt-3'.

Histone methyltransferase assay

The SET domain of TRR (residues 2,199–2,410) was expressed in *Escherichia coli* as a GST fusion, and assayed for HMTase activity as described¹². Recombinant mutant histones H3 were a gift from Y. Zhang. Edman degradation was done at the KCC Protein Facility on a 477A sequencer (Applied Biosystems).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

TRR is a nuclear protein that methylates H3-K4. **a**, Structure of TRR. Shown are NR1–NR3 and NR-R, the LXXLL and the LLXXL nuclear receptor interacting motifs, respectively. PHD, PHD finger domains. Point mutations in *trr*¹ and *trr*³ are indicated; both mutations lead to a stop codon. **b**, Detection of TRR in embryo extracts by western blotting. **c**, Detection of TRR in nuclei of third instar larval salivary glands. **d–f**, Specificity of the TRR SET domain for H3-K4 methylation. **d**, Of the four core histones, TRR SET specifically methylates histone H3. [³H]methylated histones were visualized by SDS–PAGE and autoradiography. TRR SET methylated the unmodified N-terminal peptide of histone H3 (unmodified) and an N-terminal peptide trimethylated at K9 (Me-K9), but not a peptide trimethylated at K4 (Me-K4). **e**, Equal

amounts of wild-type and several lysine-mutated derivatives of histone H3 were tested (as described in **d**) for their ability to be methylated by TRR SET. Mutated lysines are indicated. Input controls stained by Coomassie blue are shown in the bottom panels of **d** and **e**. **f**, The [³H]methylated wild-type histone H3 from **d** was subjected to Edman degradation, and ³H released at each cycle was measured by scintillation counting. **g**, Trimethylation of histone H3-K4 by TRR SET. After methylation of recombinant GST–histone H3 by purified TRR SET, the sample was separated by SDS–PAGE and probed with either antibodies against histone H3 trimethylated at K4 (top) or antibodies against GST (bottom). **h**, Western blot analysis of the methylation state of H3-K4 in extracts from wild-type and *trr*¹ mutant embryos. In each lane, 15 µg of total protein extract was loaded; the bottom panel shows the loading control using antibody against Hsc70.

Figure 2.

TRR interacts genetically and associates *in vitro* and *in vivo* with components of the ecdysone receptor complex. **a**, TRR NR2 binds EcR and USP. Bacterial extracts containing GST, GST–EcR or GST–USP were passed through a column containing the HA-tagged NR2 domain of TRR (Fig. 1a). Bound material was eluted and resolved by SDS–PAGE. **b**, Association of TRR with EcR and USP *in vivo*. Embryonic nuclear extracts were subjected to immunoprecipitation (IP) with an unrelated anti-GST, anti-HA or anti-β-Gal antibody, or with anti-TRR and anti-EcR antibodies. Immunoprecipitates were analysed by western blotting with the antibodies indicated on the right. **c**, Top, immunostaining of salivary gland polytene chromosomes with antibodies against the EcR-B1 isoform (green) and TRR (red), followed by Hoechst staining for DNA (blue). Bottom, high-magnification images of part of a chromosome. **d**, Left and middle, double immunostaining of an eye imaginal disc for TRR (red) and USP (green); right, immunostaining of a similar disc for EcR-A. Arrows indicate the morphogenetic furrow. Note that most of the region anterior to the morphogenetic furrow (left) is not stained with any of the antibodies. **e**, Clonal analysis of *trr*. Defects in eye structure in *trr¹/trr¹* clones are marked

by w^+ (red); blue line on the left indicates a defect due to incomplete morphogenetic furrow progression; blue arrows indicate defects in ommatidial organization. **f**, Interaction of *trr* and *Smr* with a dominant-negative EcR mutant in the post-furrow retinal epithelium. Representative eyes were photographed through a dissecting microscope. *GMR*, *GMR* driver alone. *F645A*, *GMR* driver and EcR-F645A responder; *P [trr]/F645A*, *GMR* driver and EcR-F645A responder plus extra copy of *trr*; *Smr*^{*BG01648*}/*F645A*, *GMR* driver and EcR-F645A responder, hemizygous for a weak mutation in *Smr* (see also Supplementary Fig. 2b). **g**, Suppression of the *B¹/trr¹* phenotype by *EcR*^{*M554*}. Double-headed arrow (same length in each panel) illustrates differing extents of the defect in morphogenetic furrow progression in different mutant combinations. **h**, Complete rescue of the *trr⁴* mutant phenotype by *EcR*^{*06210*}. In *trr4/Y*, the bracket indicates defects caused by incomplete morphogenetic furrow progression; the oval encloses defects in ommatidial structure.

Figure 3.

The role of *trr* during eye development. **a**, Enhancement of the *dpp^{blk}* phenotype by *B¹* and *trr¹*. **b**, Suppression of the *hh¹/hh¹* ‘furrow-stop’ phenotype by the *trr⁺* transgene (left), and mutant phenotype caused by the transheterozygous combination of *hh^{13C}* and *trr¹* (right, arrows indicate defects seen only in the double heterozygote). **c**, **d**, Effects of *B¹* and transheterozygous *B¹* with *trr¹* (*B¹/trr¹*) on the expression in eye discs of *dpp-lacZ* (from transposon BS3.0 *dpp-lacZ*) and *hh-lacZ* (from transposon *hh^{P30}*), assayed by immunostaining with LacZ antibodies. In **c**, brackets indicate the region that is differentially affected by *trr¹* addition. **e**, Expression of *hh* RNA (blue) is decreased in *trr* null clones, indicated by the absence of the green fluorescent protein marker (light brown). Two eye

imaginal discs from the same larva are shown; in the bottom disc, *trr* null clones posterior to the morphogenetic furrow (red arrows) are indicated by black arrows. A higher magnification image of the *trr* null clones is shown on the right. **h**, Expression of *dpp-lacZ* and TRR in wild-type eye discs, assayed by double immunostaining. Note that TRR is not expressed in the morphogenetic furrow (indicated by arrows).

Figure 4.

Molecular effects of ecdysone. **a**, Glycerol gradient analysis of TRR- and EcR-containing complexes in embryo extracts. An extract from wild-type embryos was divided into two aliquots, one of which (bottom two panels) was treated for 1 h with 10^{-6} M 20HE. Each aliquot was separated by centrifugation through a glycerol gradient. Fractions were analysed by western blotting. Approximate sizes, based on calibration using patterns of known complex components, are indicated at the top. **b**, Co-immunoprecipitation of glycerol gradient fractions. Right, the bracketed fractions in **a**, from either untreated (-) or 20HE-treated (+) extract, were precipitated with antibodies against EcR-C and analysed with antibodies against TRR. Left, input amounts of TRR. **c**, RT-PCR analysis of *hh* and *BR-C* RNA in uninduced (-) and 20HE-

treated (+) S2 cells. The same sets of primers, specific to the 3' regions of the *hh* and *BR-C Z1* transcription units, were used in **c** and **e**. Primers specific to the constitutively expressed *rp49* were tested on the same RNA samples as a control. **d**, ChIP analysis of the *hh* and *BR-C Z1* promoters in S2 cells using antibodies against EcR-B1, TRR and H3 trimethylated on K4 (Me). +20HE, addition of 20HE; G, total genomic DNA; C, control ChIP without antibody. Primers used were specific to the promoter regions of *hh* and *BR-C Z1*. **e**, RT-PCR analysis of *hh*, *BR-C Z1* and *rp49* (as a control) in RNA isolated from wild-type and *trr*¹ mutant embryos, as indicated. **f**, ChIP analysis of the methylation state of K4 in histone H3 in wild-type and *trr* mutant embryos, using antibodies specific to H3 trimethylated at K4. 'Input', control PCR amplification with the *hh* primer set using 10% of the starting material before immunoprecipitation.

Table 1

Lethality in flies expressing EcR-F645A in the eye is enhanced by mutation in *trr* and suppressed by an extra copy of *trr* or mutation in *Smr*

Genotype	Classes of progeny that were scored	Percentage (number) of mutant chromosomes recovered	Percentage (number) of balancer chromosomes recovered
<i>trr</i> ¹	Females: <i>trr</i> [OR] <i>FM7C</i> /+; <i>UAS-EcR-F645A</i> / <i>GMR-GAL4</i>	3% (4)	97% (120)
<i>trr</i> ³	Females: <i>trr</i> [OR] <i>FM7C</i> /+; <i>UAS-EcR-F645A</i> / <i>GMR-GAL4</i>	<2% (0)	>98% (41)
<i>P</i> [<i>trr</i>]	Females: <i>UAS-EcR-F645A</i> / <i>GMR-GAL4</i> ; <i>P</i> [<i>trr</i>] [OR] <i>TM3</i> /+	63% (19)	37% (11)
	Males: <i>UAS-EcR-F645A</i> / <i>GMR-GAL4</i> ; <i>P</i> [<i>trr</i>] [OR] <i>TM3</i> /+	82% (58)	18% (13)
<i>Smr</i> ^{<i>BG01648</i>}	Females: <i>Smr</i> [OR] <i>FM7C</i> /+; <i>UAS-EcR-F645A</i> / <i>GMR-GAL4</i>	59% (377)	41% (266)
	Males: <i>Smr</i> [OR] <i>FM7C</i> /Y; <i>UAS-EcR-F645A</i> / <i>GMR-GAL4</i>	94% (530)	6% (33)

Data were pooled from at least ten replicates for each of the following crosses. *trr*¹: female *trr*¹/*FM7C*; *UAS-EcR-F645A*/*CyO* × male *GMR-GAL4*. As *trr*¹ is homozygous lethal (hemizygous lethal in males), only female progeny were scored. *Smr*^{*BG01548*}: female *Smr*^{*BG01648*}/*FM7C*; *UAS-EcR-F645A* × male *GMR-GAL4*. *Smr*^{*BG01648*} is a homozygous-viable presumptive hypomorph. *Smr*^{*BG01648*}/Y males are therefore predicted to have a much lower titre of SMR than are *Smr*^{*BG01648*}/+ females. *P*[*trr*]: *UAS-F645A*/*SM5*; *P*[*trr*]/*TM3* × *GMR-GAL4*. Males produce a higher titre of TRR than do females because of dosage compensation effects on the X-derived *trr* transgene. Under the conditions of our assay, expression of EcR-F645A in the GMR domain causes roughly 95% lethality before adult eclosion. Data in the balancer column are consistent with this value in all cases except for female progeny of the *Smr* cross, in which the viability of balancer-containing flies was strongly increased by an unknown mechanism. In all cases, viability was near 100% if either *UAS-EcR-F645A* or *GMR-GAL4* was absent (data not shown).