

9-2016

The Human and Drosophila ERH are Functionally Equivalent: Evidence from Transgenic Studies

Stuart Tsubota

Brockport, stsubota@brockport.edu

Theodore Ryan

The College at Brockport

Nicholas Rizzo

The College at Brockport, npr6@duke.edu

Huey Hing

The College at Brockport, hhing@brockport.edu

Follow this and additional works at: https://digitalcommons.brockport.edu/bio_facpub

 Part of the [Biology Commons](#)

Repository Citation

Tsubota, Stuart; Ryan, Theodore; Rizzo, Nicholas; and Hing, Huey, "The Human and Drosophila ERH are Functionally Equivalent: Evidence from Transgenic Studies" (2016). *Biology Faculty Publications*. 6.

https://digitalcommons.brockport.edu/bio_facpub/6

Citation/Publisher Attribution:

The publisher's version of the article can be found here: DOI: <http://dx.doi.org/10.18103/imr.v2i8.154>

This Article is brought to you for free and open access by the Department of Biology at Digital Commons @Brockport. It has been accepted for inclusion in Biology Faculty Publications by an authorized administrator of Digital Commons @Brockport. For more information, please contact kmyers@brockport.edu.

The human and Drosophila ERH are functionally equivalent: Evidence from transgenic studies.

Authors

Stuart I. Tsubota^{a, b},
Theodore Ryan^a,
Nicholas Rizzo^a,
Huey Hing^a

^aDepartment of Biology
The College at Brockport
350 New Campus Drive
Brockport, NY 14420
Phone: (585) 395-2193
Fax: (585) 395-2741
Email:
biodept@brockport.edu

^bCorresponding author
Phone: (585) 395-5759
Fax: (585) 395-2193
Email:
stsubota@brockport.edu

Abstract

The *enhancer of rudimentary*, *e(r)*, gene encodes a small highly conserved protein, enhancer of rudimentary homolog (ERH), which has been shown to have a regulatory function in cell division, Notch signaling, and cancer progression. Human and Drosophila ERH, both 104 amino acids in length, are 76% identical and 84% similar. The high sequence identity translates into nearly identical tertiary structures. Previous studies on the expression of the human and Drosophila *e(r)* genes reveal that the two genes are similarly regulated. Data in the present study using an *e(r)-eGFP* reporter gene confirm these results, showing a high expression of the reporter in the ovaries, testes, and brain. The high structural and regulatory conservation of *e(r)* and ERH argue that human and Drosophila ERH may be biochemically and functionally equivalent. To test this hypothesis, a chimeric transgene containing the Drosophila *e(r)* non-coding regions and the human *e(r)* coding region was constructed and used to establish transgenic Drosophila stocks. This transgene can rescue all of the mutant phenotypes of an *e(r)* deletion, and Drosophila stocks in which the fly ERH has been replaced with the human ERH are fully healthy and viable. These studies demonstrate that the human and Drosophila ERH are functionally equivalent, suggesting that studies on the activity of the human ERH can be done in Drosophila, where a multitude of genetic and developmental tools are available.

1. Introduction

Since its discovery (1, 2), the enhancer of rudimentary gene, *e(r)*, has been studied with respect to a role in mitosis, cell proliferation, and cancer. The *e(r)* gene was first shown to be expressed at high levels in oogenesis and in mitotic cells in the *Drosophila* embryo (2, 3, 4). The expression pattern in the embryo is very similar to that of cyclin E (5) and correlates with cells that are undergoing DNA synthesis (6). These similarities suggest a role for *e(r)* in the cell cycle and DNA replication. This association with cell proliferation and DNA synthesis was first suggested genetically, when *e(r)* was identified as a positive regulator of *rudimentary*, *r*, the gene that codes for the first three enzymes in pyrimidine biosynthesis (2, 7). The human homologue of *r*, *CAD*, is highly expressed in dividing cells (8, 9, 10). Likewise, human *e(r)* is expressed at high levels in dividing cells (11) and has been shown to regulate the splicing of the pre-mRNA of *CENP-E*, a mitotic-motor-protein gene, indicating an important mitotic function (12).

The high expression of *e(r)* in dividing cells has led to the possibility that it may have a role in cancer. This appears to be the case. Human *e(r)* is expressed at high levels in cancerous tissue vs. normal tissue (11). In particular, *e(r)* expression increases with the tumorigenic progression of human breast cancer and ovarian cancer cells, which has led to the hypothesis that ERH expression may be diagnostic for human cancer progression (11). It also presents the intriguing possibility that high levels of ERH are necessary for tumor progression. The necessity for *e(r)* expression in cancer has been examined with RNAi approaches. In mRNA knockdown experiments with shRNA, *e(r)* was identified as a gene whose expression

was necessary for the proliferation of pancreatic, breast, and ovarian cancer cell lines (13). Similarly, *e(r)* mRNA knockdown decreased the viability of certain colorectal cancer cells with activated RAS mutations (12). This study also showed that lower ERH expression correlated with increased survival of patients with tumors carrying activated RAS mutations. Together these studies reveal that *e(r)* activity is necessary for the proliferation of certain types of cancers.

Besides the conserved expression in dividing cells, other patterns of the expression of *e(r)* may be conserved. *E(r)* is expressed at high levels in developing ovaries in *Drosophila* (3). In humans, *e(r)* is also expressed at high levels in ovaries as well as testes (11). Similarly, in the mouse, *Mus musculus*, a microarray analysis of genes expressed in the developing embryonic ovaries and testes showed that among transcripts *e(r)* ranked in the 97th percentile for expression in the ovary and the testes (14, 15). Thus *e(r)* is being expressed at high levels in the ovaries in three distantly related taxa, and in the testes in humans and mice.

The protein encoded by *e(r)* has been named ERH, enhancer of rudimentary homolog. It is a small highly conserved protein (2, 16, 17). The *Drosophila melanogaster* ERH is 76% identical to the vertebrate ERH, 49% identical to the nematode ERH, and 40% identical to the Arabidopsis ERH. A high sequence conservation is seen within the vertebrates, with the human and mouse protein being identical and differing from the zebrafish protein by a single conservative amino acid change (valine to isoleucine) (17).

The similarities in the expression of the human and *Drosophila e(r)* genes and the high conservation in the primary structure of the ERH, suggest that ERH may

be biochemically and functionally equivalent in both species. In this paper we examine this hypothesis by first taking a closer look at the structure of the two proteins, second extending the comparison of the expression patterns, and finally using a human *e(r)* transgene to show that the human ERH can functionally replace the Drosophila ERH. These data reveal the functional equivalence of the two proteins, and support the proposal that certain aspects of the human ERH activity and its regulation can be examined using the Drosophila model system.

2. Evolutionary conservation between the human and Drosophila ERH

The human and Drosophila ERH are both 104 amino acids in length and share a 76% identity and an 83% similarity (16, 17). A closer pairwise comparison along the length of the 104 amino acids shows other striking similarities. The positions of key groups of amino acids - hydrophobic amino acids, acidic amino acids, basic amino acids, and prolines are highly conserved between the two proteins (Fig. 1 a-d). These similarities in the primary structures lead to the prediction that the human and Drosophila proteins fold into very similar tertiary structures. The mouse and human ERH (They are identical.) have been synthesized in bacteria, purified, and their tertiary structure determined (18, 19). The protein folds into a single domain with a β -sheet formed by four β -strands (Fig. 1g). This flat surface is involved in the dimerization of the protein. There are also three α -helices that contribute to the globular shape of the protein. The web

server SWISS-MODEL (20) was used to generate a predicted tertiary structure of the Drosophila ERH. SWISS-MODEL uses an evolutionary modeling approach, knowledge of protein structure, and solved tertiary structures as references, to predict unknown structures. As was expected from the conserved features of the primary structures, the predicted structure of the Drosophila ERH (Fig. 1h) is highly similar to the solved human structure (Fig. 1g). Both have the β -sheet involved in protein binding and the three α -helices.

The high similarities in the primary and tertiary structures of the human and Drosophila ERH suggest that the two proteins have similar biochemical functions and regulatory features. Two other conserved features between these two proteins support this proposal. First, two casein kinase II sites that have been shown to be important in the regulation of the Drosophila ERH (21) are also present in the human ERH (Fig. 1e). This suggests that the human ERH may be regulated by casein kinase II in a similar manner to the Drosophila ERH. Second, seven amino acids (5I, 7L, 17R, 19Y, 21D, 70L, 79Y) were shown to be highly important in the dimerization of the human ERH (18). Six out of the seven of these sites are strictly conserved between human and Drosophila (Fig. 1f). The one amino acid that is not conserved is a leucine to methionine substitution, which is a conservative substitution and could be serving the same function in the Drosophila ERH.

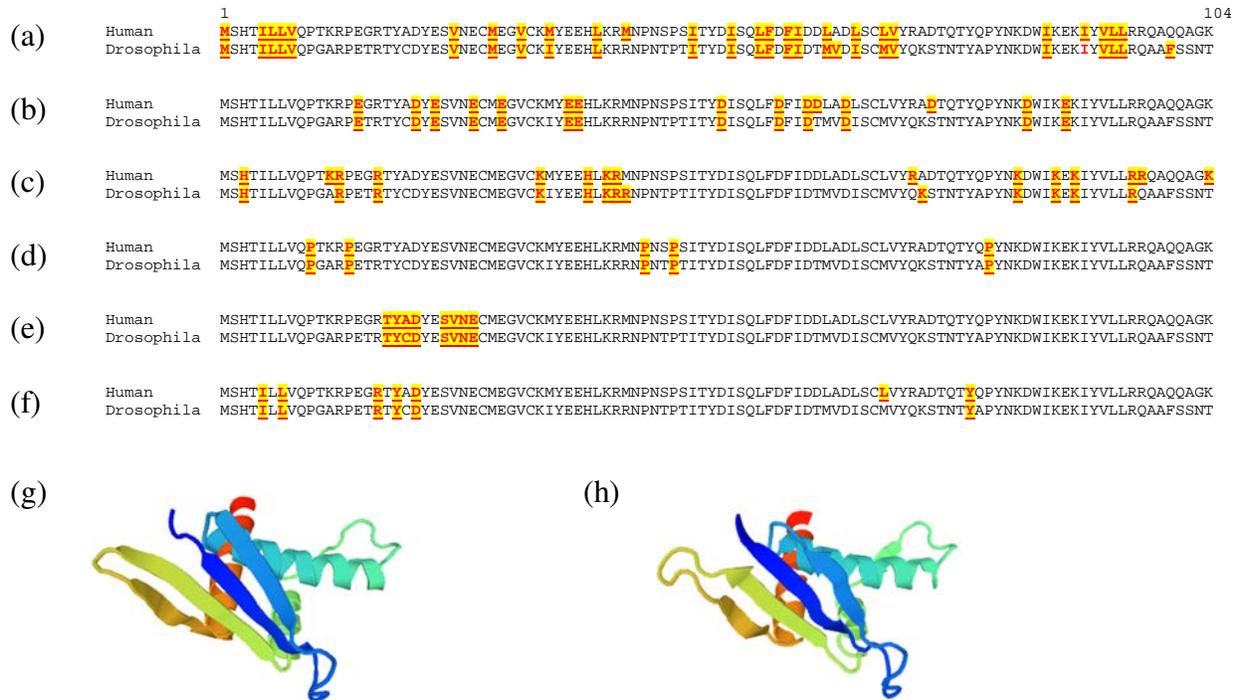


Figure 1. Comparison of the structures of human and Drosophila ERH. Various comparisons of the primary and tertiary structures of the human and Drosophila ERH are highlighted and show a high evolutionary conservation. (a) Positions of the hydrophobic amino acids (I, F, L, M, V). (b) Positions of the acidic amino acids (D, E). (c) Positions of the basic amino acids (H, K, R). (d) Position of the prolines (P). (e) Positions of two CKII phosphorylation sites, shown to be important in the regulation of ERH in Drosophila (21). (f) Positions of seven amino acids (5I, 7L, 17R, 19Y, 21D, 70L, 79Y) shown to be highly important in the dimerization of the human ERH (18). (g) Solved tertiary structure of the human ERH (R). (h) Predicted model for the tertiary structure of the Drosophila ERH.

3. Construction of transgenes used in this study

In order to examine the tissue distribution of *e(r)* and to test the functional equivalence of the human and Drosophila ERH, chimeric transgenes were constructed which consisted of the transcriptional and translational regulatory regions of the Drosophila *e(r)* gene and the protein coding regions of either the enhanced green fluorescent protein gene (*eGFP*) or the human *e(r)* gene. All of the transcriptional and translational regulatory regions of the Drosophila *e(r)* are contained within a 3.0-

kb PvuII-SalI fragment (Fig. 2a). The transgene that consists of this fragment is referred to as *ERI* in this paper, and is used as a positive control for normal *e(r)* activity. This transgene rescues the null mutant phenotypes of two *e(r)* deletions, *e(r)*²⁷⁻¹ and *e(r)*³⁷⁻⁶ (3; Tables 2-5). The regions of the *e(r)* gene that are deleted in these two mutations are shown in Fig. 2a. Both of these deletions remove the upstream region contained in *ERI* and the start of transcription. In the case of *e(r)*²⁷⁻¹, 43% of the coding region is also deleted.

The construction of the chimeric transgenes, started with the construction of

an “empty” *e(r)* gene (Fig. 2b). This gene contains all of the non-coding DNA within the PvuII-SalI fragment, but is lacking the coding region for ERH. In its place is a unique NcoI restriction-enzyme site (CCATGG). This site was chosen because using it will retain the start codon (ATG) and the Drosophila translation signal (CACC) that immediately precedes the start codon. In practice, coding regions can be synthesized or amplified with PCR to contain an NcoI-compatible site at the 5’ end and at the 3’ end, immediately following the stop codon. The PCR approach was used to amplify the human *e(r)* coding region and the *eGFP* coding region using appropriate 5’

and 3’ primers (Table 1) in the creation of the two chimeric transgenes (Fig. 2 d & e).

The significance of this approach to produce these *e(r)* transgenes, is that the inserted coding region will be regulated identically to the normal *e(r)* gene. For the *eGFP* transgene, this means that GFP can be used to examine the normal spatial distribution of ERH. For the human *e(r)* transgene, we can examine the ability of the human ERH to rescue the mutant *e(r)* phenotypes, when it is expressed at normal physiological levels, instead of over-produced levels. In this way we can more accurately compare the functionality of the human and Drosophila ERH.

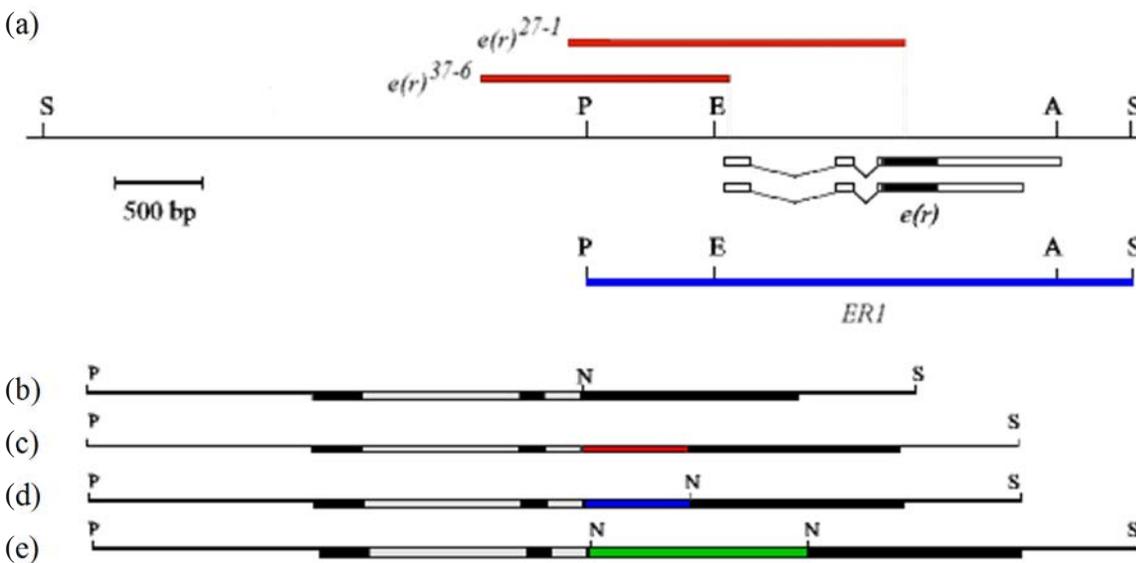


Figure 2. The wild-type Drosophila *e(r)* gene and the transgenes used in this project. (a) The 6.1-kb SalI, genomic region containing the *e(r)* gene is drawn as a horizontal line. The two transcripts of *e(r)* are indicated below the line. The extent of the two *e(r)* deletions is shown above the line. The 3.0-kb PvuII-SalI fragment that was used to create the wild-type *e(r)* transgene, *ERI*, is shown below the transcripts. (b) The “empty” *e(r)* gene used in the construction of the human *e(r)* transgene and the *eGFP* transgene. (c) Drosophila transgene, *ERI*. (d) *Hs-ER*, the human *e(r)* transgene. (e) *e(r)-eGFP*, the *eGFP* reporter gene. A = AfIII, E = EcoRV, N = NcoI, P = PvuII, and S = SalI.

Table 1. Primers used to amplify the coding regions to replace the *Drosophila e(r)* coding region

Primer	Sequence 5' to 3'
Human <i>e(r)</i> 5'	ACG <u>ACATGT</u> CTCACACCATTTTGCT
Human <i>e(r)</i> 3'	AT <u>CCATGG</u> TTATTTCCCAGCCTGTTGGGC
<i>eGFP</i> 5'	AC <u>CCATGG</u> TGAGCAAGGGCG
<i>eGFP</i> 3'	AT <u>CCATGG</u> TTACTTGTACAGCTCGTCCA

In the 5' primers, the start codon, ATG, is underlined. Human *e(r)* 5' contains a *Pci*I site, ACATGT, necessary for retaining the second codon, TCT. *eGFP* 5' contains an *Nco*I site, CCATGG, for retaining the second codon, GTG. In the 3' primers, the stop codon (reverse complement), TTA, is underlined. The human *e(r)* cDNA and the *eGFP* cDNA were used as templates in the PCR.

4. *e(r)-eGFP* expression patterns

Drosophila stocks containing the *e(r)-eGFP* reporter gene, with the *eGFP* coding region under the control of the *e(r)* transcription and translation regulatory regions (Fig. 2e), were used to determine the expression patterns of the *e(r)* gene. Three developmental stages were examined: pre-blastoderm embryos, 3rd instar larvae, and adults.

Previous studies showed a high expression of *e(r)* in adult ovaries and in the blastoderm embryos (3), so we expected to see GFP expression in these tissues. As expected there is high expression in pre-blastoderm embryos (Fig 3 a & b). This expression is the result of maternal expression of *e(r)-eGFP* and the deposition of the *GFP* mRNA and protein into the developing oocyte. While GFP is distributed throughout the early embryo, there is a higher expression at the anterior and posterior poles. This is polar localization can be seen in both pictures, but is more distinct when an embryo is photographed at a reduced exposure (Fig. 3b). While the cause of this localization is

under investigation, we believe that it is the result of the localization of the *e(r)-eGFP* mRNA in the developing oocyte. This type of localization is common in *Drosophila* oocytes. Examples are the anterior localization of the bicoid mRNA (22) and the posterior localization of the oskar mRNA (23). If the *e(r)-eGFP* mRNA is being localized to the poles of the oocyte, then that indicates that there are mRNA localization signals within the *e(r)* mRNA.

Expression of GFP in adult flies was first examined in intact individuals. Both the adult female (Fig. 3c) and male (Fig. 3d) showed high GFP expression in the head and in the abdomen. The main organ in the head is the brain, and the main organs in the abdomen are the ovaries in females and the testes in males. The abdomens were dissected and as expected, high expression was seen in the ovaries (Fig. 3e) and the testes (Fig. 3f). In the ovaries, expression appears to be higher in the early egg chambers to the right in the figure and lower in the more mature egg chambers to the left. This is consistent with the localization of *e(r)* mRNA which is present in the nurse cell of the developing egg chambers, and

deposited into the maturing oocyte (3). In the testes we see highest expression in the primary spermatocytes. The most apical region of the adult testis contains the spermatogonial cells that produce the primary spermatocytes via mitotic divisions. Adjacent to this region contains the developing primary spermatocytes, which will enter meiosis (24). This apical region has low GFP expression, but the adjacent region corresponding to developing primary spermatocytes has high GFP expression (Fig. 3f). Interestingly, studies in mice testes show that ERH is also localized to the developing spermatocytes (25).

Testes development begins in the larval stages, and by the 3rd instar larval stage they are easily visible with the

stereomicroscope through the body wall as large spheres, and are used to distinguish male and female larvae. GFP expression in whole male 3rd instar larvae can clearly be seen in the developing testes (Fig. 3g). Testes removed from the larvae show highest GFP expression in the region corresponding to primary spermatocytes (Fig. 3h). This is in agreement with the GFP expression in the adult testis (Fig. 3f).

Ovary development in female 3rd instar larvae is minimal compared to testis development in male 3rd instar larvae, so the ovaries are not visible with a normal stereomicroscope. However, a small focus of GFP expression can be seen in female 3rd instar larvae in the region where the ovaries should be present (Fig. 3i).

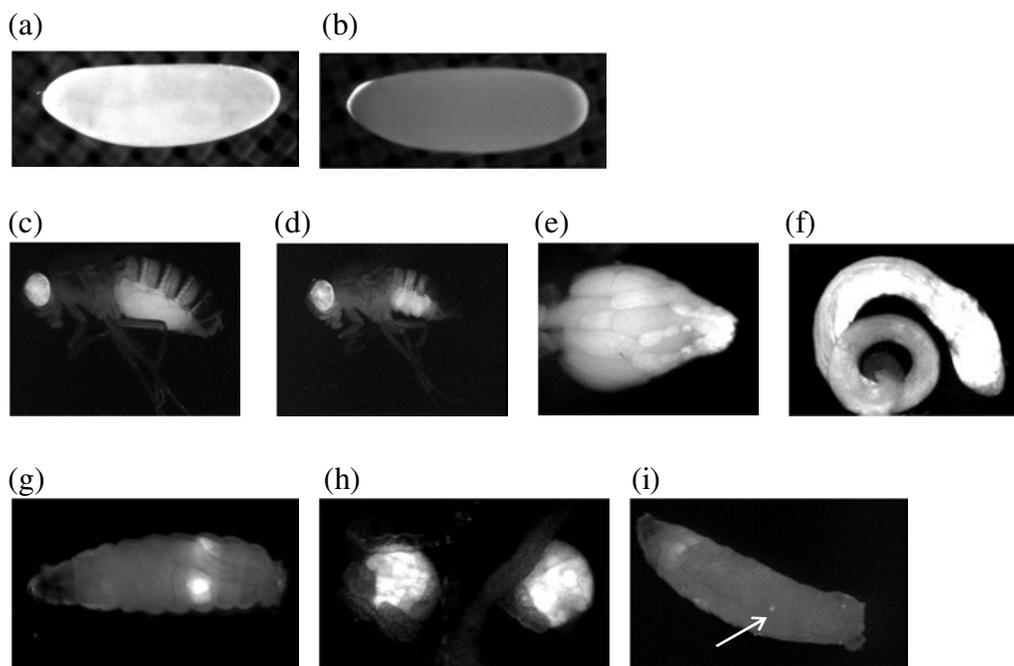


Figure 3. Localization of GFP fluorescence from *e(r)-eGFP*. (a) Pre-blastoderm embryo. Expression is throughout the embryo with higher expression at the poles. (b) Lower exposure of a pre-blastoderm embryo. Higher expression at the poles can clearly be seen. For both embryos, anterior is to the left, posterior to the right, dorsal is on the top, and ventral is on the bottom. (c) Adult female. (d) Adult male. In both adults the highest fluorescence is seen in the abdomens and the heads. For the heads, the compound eyes serve as lenses for fluorescence from the interior. (e)

Ovary from an adult female. Development of the egg chambers is from right to left with the mature oocytes to the left. Highest fluorescence is seen in the early egg chambers which are primarily composed of nurse cells that will eventually deposit their contents into the oocyte. (f) Testis from an adult male. Apical tip is at the lower right, and spermatogenesis progresses from the apical tip to the end of the coiled end of the testis. (g) Male 3rd instar larva. The two, highly fluorescing spheres inside the larva are the testes. (h) Testis from 3rd instar. The region of high fluorescence corresponds to the primary spermatocytes. This is in agreement with GFP expression in the adult testis (f). (i) Female 3rd instar larva. The ovaries are not nearly as developed as the corresponding testes; however, a fluorescent spot in the location of the developing ovary can be seen in females. It is indicated with an arrow. GFP fluorescence was imaged using a Zeiss Discovery V8 fluorescent dissecting microscope with a GFP filter.

Expression of *e(r)* in the adult Drosophila brain had not been examined previously, so it was of interest to examine *e(r)-eGFP* expression in isolated brains. In this localization study an antibody to GFP and a confocal microscope were used instead of GFP fluorescence and a dissecting microscope. Expression appears to be ubiquitous in the brain and restricted primarily to the cell bodies of the nerve cells (Fig 4a). Regions of the brain that show lower GFP expression are the synaptic regions, as shown by the antibody staining to Bruchpilot (26) a protein localized to

synaptic regions of the brain (Fig 4b). This high expression in the adult brain is consistent with the high expression of ERH in the embryonic and 3rd instar larval brain and CNS (4). Thus, as with ovaries and testes, we see a high expression in the brain throughout the development of the fly.

It should be noted that in human and mouse, *e(r)* is also expressed in the brain. In human, northern blots and dot blots showed *e(r)* mRNA in the brain (11, 27). In the mouse microarray analysis showed that of the transcripts expressed in the brain, *e(r)* ranked in the 91st percentile (15).

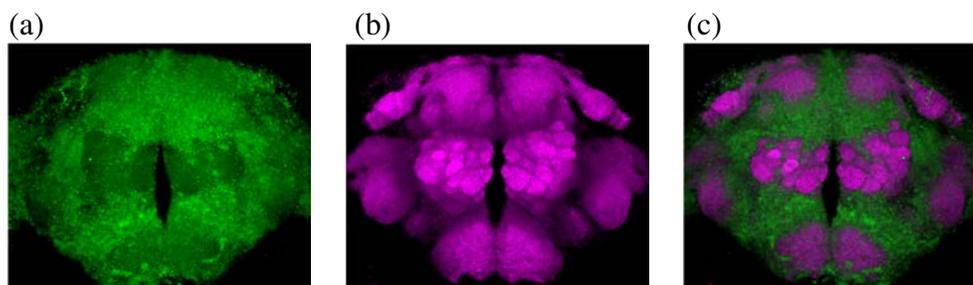


Figure 4. GFP localization in the brain of the adult fly. The images show antibody localization to the brain. The view is from the front of the fly. The compound eyes on the left and right have been removed. (a) GFP antibody localization. GFP can be seen throughout the brain, with most of the localization being in the cell bodies of the neurons. (b) Bruchpilot antibody localization (mouse nc82 monoclonal antibody). Bruchpilot localizes to synaptic junctions and thus identifies regions containing the axons of neurons. These regions coincide with the regions of lower

GFP staining. (c) Superimposed GFP and Bruchpilot localization. The brains were imaged at 630x magnification using a Zeiss LSM 700 confocal microscope.

5. Testing the functionality of the human ERH in Drosophila

The evolutionary conservation of the structures of the human and Drosophila ERH (Fig. 1) and the conserved aspects of the expression of the gene (Fig. 3 & 4), strongly suggest that the two proteins are functionally equivalent. This possibility is supported by the fact that in human cells, the Drosophila ERH expressed from a Drosophila *e(r)* transgene, is nuclearly localized like its human counterpart (28). This means that Drosophila ERH must successfully interact with the nuclear localization system of the human ERH. In addition, the human and Drosophila ERH, both have RPS3 as a binding partner, and the Drosophila ERH can successfully interact with the human RPS3 in a yeast two-hybrid assay (28).

To test the functional equivalence of the human and Drosophila ERH, we constructed a human *e(r)* transgene that contains all of the non-coding regulatory regions of the Drosophila *e(r)* gene with the coding region of the human *e(r)* gene (Fig. 2d). The significance of this construction is that it ensures that the human *e(r)* coding region will be expressed in an identical pattern to the normal Drosophila *e(r)* gene, and importantly at normal levels, rather than over-expressed levels. This chimeric *e(r)* gene was used to transform *Drosophila melanogaster*, and stocks containing an autosomal transgene were established. These transgenic stocks were used to test the ability of the human ERH to rescue the mutant phenotypes of an *e(r)* null mutation. Four different mutant phenotypes were utilized: 1) lethality of the $e(r)^{27-1} r^{hd1}$ double mutant, 2) lethality of the $e(r)^{37-6} N^{mid-p}$ double mutant, 3) low fecundity of $e(r)^{27-1}$

females, and 4) low viability of the $e(r)^{27-1}$ mutant. The two *e(r)* alleles used in this study are both deletions and act as *e(r)* null alleles (3, Fig. 2a). For the rescue experiments, crosses were performed to produce flies which were missing the Drosophila *e(r)* gene, but which had the human *e(r)* transgene. As a positive control in all of the tests, stocks containing *ERI*, the *Drosophila melanogaster e(r)* transgene, were used to show rescue by the *Drosophila melanogaster e(r)* gene.

6. The human *e(r)* transgene rescues the lethality of the $e(r)^{27-1} r^{hd1}$ double mutant.

The original *e(r)* mutant allele, $e(r)^{p1}$, was isolated as an enhancer of the mutant wing phenotype of a rudimentary allele, r^{hd1} (1, 2). It turned out $e(r)^{p1}$ was a leaky *e(r)* mutation, and that the combination of an *e(r)* null with r^{hd1} has a very low viability (Table 2). Both *e(r)* and *r* are X-linked genes so the lethality of the $e(r)^{27-1} r^{hd1}$ double mutant can be assessed by the absence of the hemizygous males. Rescue of the lethality is seen by the presence of the double mutant males with the transgene. Heterozygous females from the crosses are used as a control for normal viability. As can be seen in Table 2, two different human *e(r)* transgenes can rescue the double mutant lethality. As a control, the equivalent *Drosophila melanogaster e(r)* transgene, *ERI*, also rescues the double mutant lethality. The Male/Female ration shows that all three transgenes show good rescue. The lower than wild-type viability of these males is caused by the r^{hd1} mutation. The rescue in these experiments show that the human ERH can interact successfully with rudimentary and pyrimidine metabolism in Drosophila.

Table 2. Rescue of lethality of $e(r)^{27-1} r^{hd1}$

Genotype Tested	$e(r)^{27-1} r^{hd1}/+$ Females	$e(r)^{27-1} r^{hd1}$ Males	Male/Female
$e(r)^{27-1} r^{hd1}$	642	0	0.00
$e(r)^{27-1} r^{hd1}; ERI$	165	37	0.22
$e(r)^{27-1} r^{hd1}; Hs-ER 4F$	297	110	0.37
$e(r)^{27-1} r^{hd1}; Hs-ER 1F$	196	61	0.31

7. The human $e(r)$ transgene rescues the lethality of the $N^{nd-p} e(r)^{37-6}$ double mutant.

The weak *Notch* allele, N^{nd-p} , was isolated in a lethal interaction with a weak $e(r)$ allele, $e(r)^{p2}$ (4). In the background of $e(r)^+$, N^{nd-p} mutants show good viability, but the double mutant combinations with leaky or null $e(r)$ alleles are lethal (4). Since both N and $e(r)$ are X-linked genes, lethality of the double mutants is seen as the absence of hemizygous males (Table 3). Heterozygous females are used as a control. Full viability of the hemizygous males would produce equal numbers of males and females. The $N^{nd-p} e(r)^{37-6}$ double mutant is lethal. As a

negative control the $e(r)$ -*eGFP* transgene (Fig. 2e) used in the expression studies (Fig. 3 & 4) did not rescue the lethality (Table 3). Two different stocks carrying the *Hs-ER* transgene were used in this study, and in both cases the lethality of $N^{nd-p} e(r)^{37-6}$ was rescued. The extent of the rescue as seen by the Male/Female rescue was similar to that seen with the *Drosophila melanogaster e(r)* transgene, *ERI* (Table 3). The lower than wild-type viability of the transgenic males is caused by the N^{nd-p} mutation. The rescue in these experiments show that, in the interaction with the Notch signaling pathway in *Drosophila*, the human ERH functions normally.

Table 3. Rescue of lethality of $N^{nd-p} e(r)^{37-6}$

Genotype Tested	$N^{nd-p} e(r)^{37-6}/+$ Female	$N^{nd-p} e(r)^{37-6}$ Male Progeny	Male/Female
$N^{nd-p} e(r)^{37-6}$	656	0	0.00
$N^{nd-p} e(r)^{37-6}; e(r)$ - <i>eGFP</i>	389	0	0.00
$N^{nd-p} e(r)^{37-6}; ERI$	148	38	0.26
$N^{nd-p} e(r)^{37-6}; Hs-ER 4F$	293	66	0.22
$N^{nd-p} e(r)^{37-6}; Hs-ER 4M$	98	33	0.34

8. The human $e(r)$ transgene rescues the low fecundity of $e(r)^{27-1}$ females.

There is high expression $e(r)$ in the developing ovaries (3, Fig. 3e). Thus it is not unexpected that $e(r)$ null females

produce very few offspring. To test the ability of the human $e(r)$ transgene to rescue the low fecundity of the $e(r)^{27-1}$ females, stocks were constructed that were homozygous for both $e(r)^{27-1}$ and an autosomal insertion of the human $e(r)$

transgene or the Drosophila *e(r)* transgene, *ERI*. Ten females of each stock were crossed to ten wild-type males, and allowed to lay eggs for ten days. The total number of adult progeny produced from these crosses was used as a measure of fecundity. Both human *e(r)* transgenes rescue the low

fecundity at levels comparable to that of the Drosophila *e(r)* transgene (Table 4). These data indicate that the human ERH can perform the function in Drosophila that are necessary to produce normal female fecundity. This requires activity in the normal development of the ovaries.

Table 4. Rescue of low fecundity of *e(r)* null females

Genotype of females	Total Progeny
<i>e(r)²⁷⁻¹</i>	21
<i>e(r)²⁷⁻¹; ERI</i>	620
<i>e(r)²⁷⁻¹; Hs-ER 1F</i>	1027
<i>e(r)²⁷⁻¹; Hs-ER 1M</i>	748

9. The human *e(r)* transgene rescues the low viability of *e(r)²⁷⁻¹ mutants.*

While null mutants of *e(r)* are not lethal, they have a low a low viability (3). This low viability is probably the result of the lack of *e(r)* activity in a number of different tissues. To test the ability of the human *e(r)* transgene to rescue this low viability, crosses were set up to produce *e(r)²⁷⁻¹* hemizygous males with or without the *e(r)* transgene. If the *e(r)* transgene does not rescue the low viability, then these two groups of *e(r)²⁷⁻¹* males should be present in equal amounts and in low numbers. If the *e(r)* transgene rescues the low viability, then the transgene containing males should be significantly greater in number than the males without the transgene. As a control for wild-type viability the *e(r)^{27-1/+}*,

transgene containing females from the crosses were counted. If the *e(r)²⁷⁻¹*, transgene containing males have wild-type viability, then their numbers should approximate the number of these females. The human *e(r)* transgene rescued the low viability of the *e(r)²⁷⁻¹* males (Table 5). The number of transgene containing males was much greater than those of the *e(r)²⁷⁻¹* males without the transgene, and were close to the numbers of the control females. As a positive control, the Drosophila *e(r)* transgene also showed rescue of the low viability of *e(r)²⁷⁻¹* males, at a similar level to the rescue seen by the human *e(r)* transgene. In both cases the number of transgene containing males was about six times that of the males without the transgene.

Table 5. Rescue of the low viability of *e(r)²⁷⁻¹ mutants*

Genotype Tested	<i>e(r)^{27-1/+}</i> ; transgene Females	<i>e(r)²⁷⁻¹</i> ; transgene Males	<i>e(r)²⁷⁻¹</i> Males	males with transgene/ males without transgene
<i>e(r)²⁷⁻¹; ERI</i>	160	194	32	6.06
<i>e(r)²⁷⁻¹; Hs-ER 1F</i>	278	245	41	5.98

10. Conclusions and Discussion

The different *e(r)* mutant phenotypes reveal the roles of *e(r)* in different aspects of *Drosophila* development and physiology and in different biochemical and signaling pathways. The rescue of all of these mutant phenotypes by the human *e(r)* transgene, at levels comparable to the *Drosophila e(r)* transgene, indicates that the human ERH can functionally replace the *Drosophila* ERH throughout the development of the fruit fly. In fact, we now maintain viable and healthy stocks of *Drosophila melanogaster* in which the *Drosophila e(r)* has been deleted and replaced with the human *e(r)* transgene used in this study. This means that we have not been able to identify any *e(r)* loss-of-function phenotypes that cannot be rescued by the human ERH. Thus, the human and *Drosophila* ERH are functionally equivalent within the fruit fly, *Drosophila melanogaster*.

There are other data that argue that the human and *Drosophila* ERH are performing similar functions. In *Drosophila*, its initial isolation as a regulator of pyrimidine biosynthesis and its

expression in dividing cells pointed to a role in DNA synthesis and mitosis (1, 2). This has also been noted in humans, where *e(r)* expression is associated with dividing cells, specifically cancer cells (11), indicating a possible function in cancer progression. Consistent with this proposal is the evidence that down-regulation of *e(r)* by RNAi inhibits the progression of certain cancers (12, 13), and low *e(r)* expression correlates with higher survival among patients with certain cancers (12). Understanding the activity and regulation of ERH will be important in defining its role in cancer. Given the functional equivalence of the human and *Drosophila* ERH, the various *e(r)* mutant phenotypes, and the assays for wild-type ERH activity in *Drosophila*, the *Drosophila melanogaster* system will be useful in dissecting human ERH activity.

11. Acknowledgements

This work was supported by a grant from the National Institutes of Health (R15 GM64364), and by funds from The College at Brockport.

The College at Brockport

12. References

1. Tsubota, S. I. & Schedl, P. (1986). Hybrid dysgenesis-induced revertants of insertions at the 5' end of the rudimentary gene in *Drosophila melanogaster*: transposon-induced control mutations. *Genetics*, 114, 165-182.
2. Wojcik, E., Murphy, A. M., Fares, H., Dang-Vu, K., & Tsubota, S. I. (1994). Enhancer of rudimentary^{pl}, *e(r)*^{pl}, a highly conserved enhancer of the rudimentary gene. *Genetics*, 138, 1163-1170.
3. Roykhman, M., Ibach, S., Harding, D., Wolff, B., Vowels, M., Gelsthorpe, M. E., . . . Tsubota, S. I. (2007). The generation and analysis of deficiencies within a small genomic region on the X chromosome of *Drosophila melanogaster* containing two genes,

- enhancer of rudimentary* and *CG15352*. *Fly*, 1, 245-250.
4. Tsubota, S. I., Vogel, A. C., Phillips, A. C., Ibach, S. M., Weber, N. K., Kostrzebski, M. A., & Spencer, S. A. (2011). Interactions between *enhancer of rudimentary* and *Notch* and *deltex* reveal a regulatory function of *enhancer of rudimentary* in the Notch signaling pathway in *Drosophila melanogaster*. *Fly*, 5, 1-10.
 5. Knoblich, J. A., Sauer, K., Jones, L., Richardson, H., Saint, R., & Lehner, C. F. (1994). Cyclin E controls S phase progression and its down-regulation during *Drosophila* embryogenesis is required for the arrest of cell proliferation. *Cell*, 77, 107-120.
 6. Hao, X. F., Alphey, L., Bandara, L. R., Lam, E.W.-F., Glover, D., & La Thangue, N. B. (1995). Functional conservation of the cell cycle-regulating transcription factor DRTF1/E2F and its pathway of control in *Drosophila melanogaster*. *J Cell Sci*, 108, 2945-2954.
 7. Rawls, J. M. & Fristrom, J. (1975). A complex genetic locus that controls the first three steps of pyrimidine biosynthesis in *Drosophila*. *Nature*, 255, 738-740.
 8. Aoki, T. & Weber, G. (1981). Carbamoyl phosphate synthetase (glutamine hydrolyzing): increased activity in cancer cells. *Science*, 212, 463-465.
 9. Aoki, T., Morris, H., & Weber, G. (1982). Regulatory properties and behavior of activity of carbamoyl phosphate synthetase (glutamine hydrolyzing) in normal and proliferating tissues. *J Biol Chem*, 257, 432-438.
 10. Rao, G. & Davidson, J. (1988). *CAD* gene expression in serum-starved and serum-stimulated hamster cell. *DNA*, 7, 423-432.
 11. Zafrakas, M., Losen, I., Knüchel, R. & Dahl, E. (2008). Enhancer of the rudimentary gene homologue (*ERH*) expression pattern in sporadic human breast cancer and normal breast tissue. *BMC Cancer*, 8, 145. DOI: 10.1186/1471-2407-8-145.
 12. Weng, M. T., Lee, J. H., Wei, S. C., Li, Q., Shahamatdar, S., Hsu, D., . . . Luo, J. (2012). Evolutionarily conserved protein ERH controls CENP-E mRNA splicing and is required for the survival of KRAS mutant cancer cells. *Proc Natl Acad Sci USA*, 109, E3659–E3667.
 13. Marcotte, R., Brown, K. R., Suarez F., Sayad, A., Karamboulas, K., Krzyzanowski, P. M., . . . Moffat, J. (2011). Essential gene profiles in breast, pancreatic, and ovarian cancer cells. *Cancer Discovery*, 2, 172-189.
 14. Bouma, G. J., Affourtit, J. P., Bult, C. J., & Eicher, E. M. (2007). Transcriptional profile of mouse pre-granulosa and Sertoli cells isolated from early-differentiated fetal gonads. *Gene Expr Patterns*, 7, 113-23.
 15. ERH - various normal tissues. <https://www.ncbi.nlm.nih.gov/geoprofiles/47380842>
 16. Isomura, M., Okui, K., Fujiwara, T., Shin, S., & Nakamura, Y. (1996). Cloning and mapping of a novel human cDNA homologous to DROER, the enhancer of the *Drosophila melanogaster* rudimentary gene. *Genomics*, 32, 125–127.
 17. Gelsthorpe, M., Pulumati, M., McCallum, C., Dang-Vu, K., & Tsubota, S. I. (1997) The putative cell

- cycle gene, *enhancer of rudimentary*, encodes a highly conserved protein found in plants and animals. *Gene*, 186, 189-195.
18. Arai, R., Kukimoto-Niino, M., Uda-Tochio, H., Morita, S., Uchikubo-Kamo, T., Akasaka, R., . . . Yokoyama, S. (2005). Crystal structure of an enhancer of rudimentary homolog (ERH) at 2.1 Å resolution. *Protein Sci*, 14, 1888–1893.
 19. Wan, C., Tempel, W., Liu, Z. J., Wang, B. C., & Rose, R. B. (2005). Structure of the conserved transcriptional repressor enhancer of rudimentary homolog. *Biochemistry*, 44, 5017–5023.
 20. Biasini, M., Bienert, S., Waterhouse, A., Arnold, K., Studer, G., Schmidt, T., . . . Schwede, T. (2014). SWISS-MODEL: modelling protein tertiary and quaternary structure using evolutionary information. *Nucleic Acids Research*, 42 (W1), W252-W258, doi: 10.1093/nar/gku340
 21. Gelsthorpe, M. E., Tan, Z., Phillips, A., Eissenberg, J. C., Miller, A., Wallace, J., & Tsubota S. I. (2006). Regulation of the *Drosophila melanogaster* protein, Enhancer of Rudimentary, by casein kinase II. *Genetics*, 174, 265-270.
 22. Berleth, T., Burri, M., Thoma, G., Bopp, D., Riehlstein, S., Frigerio, G., Noll, M., & Nüsslein-Volhard, C. (1988). The role of localization of bicoid RNA in organizing the anterior pattern of the *Drosophila* embryo. *EMBO J*, 7, 1749–1756.
 23. Ephrussi, A., Dickinson, L. K., & Lehmann, R. (1991). Oskar organizes the germ plasm and directs localization of the posterior determinant nanos. *Cell*, 66, 37–50.
 24. Fuller, M. (1992). Spermatogenesis. In M. Bate and A. Martinez Arias (Eds.), *The Development of Drosophila melanogaster* (pp. 71-147). Plainview, NY: CSHL Press.
 25. Sutherland, J. M., Sobinoff, A. P., Fraser, B. A., Redgrove, K. A., Davidson, T.-L., Siddall, N. A., . . . McLaughlin, E. A. (2015). RNA binding protein Musashi-1 directly targets Msi2 and Erh during early testis germ cell development and interacts with IPO5 upon translocation to the nucleus. *The FASEB Journal*, 29, 2759-2768.
 26. Wagh, D. A., Rasse, T. M., Asan, E., Hofbauer, A., Schwenkert, I., Dürrbeck, H., . . . Buchner, E. (2006). Bruchpilot, a protein with homology to ELKS/CAST, is required for structural integrity and function of synaptic active zones in *Drosophila*. *Neuron*, 49, 833-44.
 27. Smyk, A., Szuminska, M., Uniewicz, K. A., Graves, L. M., & Kozłowski, P. (2006). Human enhancer of rudimentary is a molecular partner of PDIP46/SKAR, a protein interacting with DNA polymerase δ and S6K1 and regulating cell growth. *FEBS J*, 273, 4728-4741.
 28. Phillips, A. (2005). *Genetic and Biochemical Studies of Enhancer of Rudimentary to Elucidate Protein Function*. (Unpublished doctoral dissertation). Saint Louis University, St. Louis, MO, USA.