EOR-2 is an obligate binding partner of the BTB-zinc finger protein EOR-1 in *C. elegans*

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ABSTRACT

BTB-zinc finger transcription factors play many important roles in metazoan development. In these proteins, the BTB domain is critical for dimerization and for recruiting cofactors to target genes. Identification of these cofactors is important for understanding how BTB-zinc finger proteins influence transcription. Here we show that the novel but conserved protein EOR-2 is an obligate binding partner of the BTB-zinc finger protein EOR-1 in *Caenorhabditis elegans*. EOR-1 and EOR-2 function together to promote multiple Ras/ERK-dependent cell fates during development, and we show that EOR-1 is a robust substrate of ERK in *vitro*. A point mutation (L81F) in the EOR-1 BTB domain reduces both ERK phosphorylation and EOR-2 binding and eliminates all detectable biological function without affecting EOR-1 expression levels, localization, or dimerization. This point mutation lies near the predicted charged pocket region of the EOR-1 BTB dimer, a region that, in other BTB-zinc finger proteins, has been proposed to interact with corepressors or coactivators. We also show that a conserved zinc finger-like motif in EOR-2 is required for binding to EOR-1, that the interaction between EOR-1 and EOR-2 is direct, and that EOR-2 can bind to the human BTB-zinc finger protein PLZF. We propose that EOR-2 defines a new family of cofactors for BTB-zinc finger transcription factors that may have conserved roles in other organisms.
INTRODUCTION

The BTB (or POZ) domain, named for founding members bric-à-brac, tramtrack, and broad complex, is a protein-protein interaction domain evolutionarily conserved from yeast to humans (reviewed in PEREZ-TORRADO et al. 2006). While some proteins such as Skp1 and Elongin C are made up almost entirely of the BTB domain, most BTB proteins consist of the BTB domain in the N terminus and other functional domains in the C terminus by which they are classified. These different classes of BTB domains all share a well-conserved tertiary structure termed the “core BTB fold” (STOGIOS et al. 2005). The BTB fold contains exposed surfaces that are quite variable in sequence between the different classes of BTB-containing proteins, thus allowing for a diverse range of protein-protein interactions. BTB-containing proteins have roles in a variety of biological processes including transcriptional regulation (DAVID et al. 1998; DEWEINDT et al. 1995; KERRIGAN et al. 1991), protein degradation (BAI et al. 1996; LONERGAN et al. 1998; PINTARD et al. 2003), cytoskeleton organization (BOMONT et al. 2000; HARA et al. 2004; ROBINSON and COOLEY 1997), and ion transport (MINOR et al. 2000). Despite their diverse functions, these proteins all use their BTB domain for self-association or for interactions with other proteins; thus, identification of partners that bind the BTB domain is often important for understanding a protein’s function.

The BTB-zinc finger protein family (or POK family for POZ and Kruppel) is one class of BTB-containing proteins that primarily functions as transcriptional regulators. These proteins bind to specific DNA sequences in target gene promoters through their Kruppel-like C2H2 zinc fingers and dimerize and/or recruit co-regulators to these sites through their BTB domains (reviewed in COSTOYA 2007). Many BTB-zinc finger proteins, including the human oncoproteins PLZF and BCL-6, function as transcriptional repressors and use their BTB domains
to recruit co-repressors such as N-Cor, SMRT, mSin3A, and HDAC-1, and/or Polycomb group proteins (Barna et al. 2002; Gearhart et al. 2006; reviewed in Prive et al. 2005). Some BTB-zinc finger repressors bind other transcription factors to prevent their activation of target genes (Lee et al. 2002; Pagans et al. 2004). A few BTB-zinc finger proteins, including Drosophila GAGA factor, also have the ability to activate transcription (Biggin and Tjian 1988; Rodova et al. 2004; Staller et al. 2001). Recently, the GAGA factor BTB domain was shown to bind to the TAFIID general transcription factor subunit TAF-3, suggesting the GAGA factor may function as a transcriptional activator by recruiting the core TAFIID machinery to its target promoters (Chopra et al. 2008). In general, however, much less is known about protein interactions of the BTB domain in BTB-zinc finger proteins that function as activators.

EOR-1 is one of only two BTB-zinc finger proteins in C. elegans (Stogios et al. 2005), making the worm a simple model in which to study BTB-zinc finger proteins. The overall structure of EOR-1 is similar to PLZF: a BTB domain in the N terminus and 9 similarly spaced C2H2 zinc finger domains in the C terminus (depicted in Fig. 1A and Howard and Sundaram 2002; Hoeppner et al. 2004). In addition to these domains, EOR-1 contains a BACK domain and a polyglutamine stretch. BACK domains are typically found immediately following the BTB domain in BTB-Kelch proteins but their function is still unclear (Stogios and Prive 2004). Polyglutamine domains are found in several transcriptional regulators including the BTB-zinc finger protein GAGA in which it acts as a transactivation domain (Vaquero et al. 2000). It is not yet clear whether EOR-1 functions as a transcriptional repressor and/or activator, as none of its direct targets are known.

EOR-1 was identified in a forward genetic screen for components of the C. elegans Ras/ERK signaling pathway (Rocheleau et al. 2002). EOR-2, a novel protein, was also
identified in this screen, and as such, is a potential partner of EOR-1 (HOWARD and SUNDARAM 2002; ROCHELEAU et al. 2002). EOR-2 is not highly related to other proteins outside of nematodes, but Contrast Hierarchical Alignment and Interaction Network (CHAIN) analysis revealed that its C-terminus shares weak similarity with uncharacterized proteins from other organisms, including *Drosophila* and humans (HOEPPNER et al. 2004). This region of similarity includes a CHHC zinc finger-like motif, albeit with atypical spacing. Thus, EOR-2 may have a conserved function.

Loss of function mutations in *eor-1* and in *eor-2* cause incompletely penetrant defects in several cell types whose fates depend on the Ras/ERK signaling pathway, including the excretory duct cell, the P12 ectoblast, and vulval precursor cells (VPCs) (HOWARD and SUNDARAM 2002; ROCHELEAU et al. 2002). Epistasis experiments suggest that EOR-1 and EOR-2 act downstream or in parallel to MPK-1/ERK and function redundantly with the known MPK-1 target LIN-1, an Ets family transcription factor (BEITEL et al. 1995; HOWARD and SUNDARAM 2002; JACOBS et al. 1998). *eor-1* mutations also show strong genetic interactions with the Hox genes *egl-5* and *lin-39* (HOWARD and SUNDARAM 2002), which are downstream targets of the Ras/ERK pathway in P12 and the VPCs, respectively (EISENMANN et al. 1998; JIANG and STERNBERG 1998; MALOOF and KENYON 1998), and *eor-1* mutants have reduced EGL-5 protein expression in the P12 lineage (HOWARD-BARFIELD 2004). Taken together, these experiments suggest that EOR-1 and EOR-2 also may be targets of ERK or may cooperate with the Ras/ERK pathway to transcriptionally upregulate Hox gene expression.

EOR-1 and EOR-2 are predicted to function together based on several genetic arguments (HOWARD and SUNDARAM 2002). First, *eor-1* mutants are identical to *eor-2* mutants in phenotype and in genetic interactions with other Ras/ERK pathway mutants. Second, based on
reporter gene studies, both proteins are ubiquitously expressed and nuclear-localized. Finally, *eor-1; eor-2* double mutant phenotypes are no more severe than those of either single mutant, suggesting both genes function together in a common biological process. In addition, *eor-1* and *eor-2* have roles outside of Ras/ERK signaling; both genes were also identified in screens for defects in cell death of hermaphrodite-specific neurons (HSNs) and for defects in phasmid dye uptake (HOEPPNER et al. 2004). *eor-1* and *eor-2* single and double mutants are identical with respect to these phenotypes, suggesting again EOR-1 and EOR-2 function together. Based on transcriptional and translational GFP reporters, EOR-1 and EOR-2 do not appear to regulate each other’s expression or localization (HOEPPNER et al. 2004; HOWARD-BARFIELD 2004). Furthermore, over-expression of EOR-1 can not compensate for loss of EOR-2 function and vice versa (HOWARD-BARFIELD 2004). Therefore, it is unclear what the relationship between EOR-1 and EOR-2 is and how the two proteins function together to positively mediate Ras/ERK signaling.

Here we show that EOR-1 and EOR-2 are obligate binding partners *in vivo*, and that the interaction between EOR-1 and EOR-2 requires the BTB domain of EOR-1 and the conserved CHHC domain of EOR-2. In addition, we show that EOR-1 is a substrate of ERK *in vitro*, further supporting the idea that EOR-1 may be a direct target of ERK signaling *in vivo*.

**MATERIALS AND METHODS**

**Strains:** All strains were maintained by standard methods unless noted otherwise. Bristol N2 was used as the wild-type strain. The following alleles were used: *eor-1(cs28), eor-1(cs44), eor-2(cs30), sos-1(cs41)* (ROCHELEAU et al. 2002); *lin-39(n709)* (described in RIDDLE et al. 1997).
**Plasmid construction:** All Gal4DBD-fusion constructs were made by inserting cDNAs into pCMX-Gal4DBD (gift from Mitch Lazar, University of Pennsylvania). All 3xFLAG constructs were made by inserting cDNAs into p3XFLAG-myc-CMV-24 (Sigma). All GST constructs were made by inserting cDNAs into pGEX-4T1 (GE Healthcare). *eor-1p::EOR-1::GFP* and *eor-2p::EOR-2::GFP* were previously described (Howard and Sundaram 2002). Site-directed mutagenesis of these constructs was used to create *eor-1p::EOR-1L81F::GFP, eor-1::EOR-1S64A::GFP, eor-1::EOR-1S248A::GFP, eor-1::EOR-1S64A;S248A::GFP, eor-1::EOR-1S64A;S87A;S248A::GFP, and eor-2p::EOR-2C900S::GFP.* To generate the *lin-31p* expression constructs, the *lin-31p* fragment was digested from pB255 (TAN et al. 1998) and inserted into pPD49.26 (Addgene) to generate pKH20. Site-directed mutagenesis was performed to add a *NotI* site in the multiple cloning site of pKH20 to generate pKH22. *eor-1* and *eor-2* cDNA was inserted into the *NotI* site of pKH22 to generate *lin-31p::EOR-1* and *lin-31p::EOR-2*, respectively. To generate the *dpy-7p* expression constructs, the *dpy-7p* fragment was digested from *dpy-7::2xNLS::yfp* (MYERS and GREENWALD 2005) and inserted into pPD49.26 (Addgene) to generate pKH11. *eor-1* and *eor-2* cDNA was inserted into pKH22 to generate *dpy-7p::EOR-1* and *dpy-7p::EOR-2*, respectively. Details of all plasmid constructions are available upon request.

**Antibodies for C. elegans Western blots:** A polyclonal peptide antibody directed against amino acids 894-909 of EOR-1 was generated by Zymed Laboratories. Before use, the EOR-1 antibody was preabsorbed with acetone powder made from *eor-1(cs28)* animals and diluted 1:2,000. The anti-HSP90 antibody, a gift from Johji Miwa and Yasunori Yamaguchi, was previously described (INOUE et al. 2003) and used at 1:1,000 to control for loading. The MEL-26
antibody, a gift from Paul Mains, was previously described (PINTARD et al. 2003) and used at 1:500. The anti-HDA-1 antibody (Santa Cruz sc-5550) was used at 1:1,000. Anti-mouse and anti-rabbit secondary antibodies conjugated to peroxidase (GE Healthcare) were used at 1:20,000.

**C. elegans Western blotting:** Staged L4 animals were washed from NGM plates in M9 and lysed using a glass dounce homogenizer in buffer (50mM Tris-HCl, pH7.5, 150mM NaCl, 1.5mMgCl₂, 0.5% NP-40) containing protease inhibitors. Extracts were centrifuged to pellet debris and the concentration of the supernatant was determined by Bradford assay (Biorad). For each strain, equal amounts of protein were separated by SDS-PAGE, transferred to nitrocellulose membrane, and probed with the EOR-1 antibody. Following detection with a chemiluminescent substrate kit (Pierce), blots were then re-probed with the HSP90 antibody to check for equal loading.

**293T cell immunoprecipitations:** 293T cells were grown to 80% confluency in 6-well culture plates containing DMEM supplemented with 10% FBS, penicillin, and streptomycin. Prior to transfection, media was removed and replaced with media lacking antibiotics. Cells were transfected with 4μg of the indicated constructs using Lipofectamine 2000 (Invitrogen) following the manufacturer’s protocol. Cells were collected and lysed 24 hours later in buffer (50mM KCl, 20mM HEPES, 2mM EDTA, 0.1% NP-40, 10% glycerol, 0.5% non-fat dry milk) containing protease inhibitors. Lysates were precleared with Protein G agarose (Invitrogen) for 1 hour at 4°C and then incubated with anti-Gal4 DBD-conjugated agarose (Santa Cruz RK5C1) overnight at 4°C. Immunoprecipitates were washed 4 times in lysis buffer, boiled in 2X SDS sample buffer,
and analyzed by Western blot with FLAG (Sigma A-8592) and GAL4DBD (Santa Cruz sc-510HRP) antibodies diluted 1:5,000 and 1:2,000 respectively.

**C. elegans immunoprecipitations:** Transgenic worms expressing either *csEx126 (eor-1p::EOR-1::GFP; ttx-3p::GFP), csEx122 (eor-2p: EOR-2::GFP; ttx-3p::GFP), or csEx111 (ttx-3p::GFP)* were washed from NGM plates in M9 and lysed using a glass dounce homogenizer in buffer (50mM Tris-HCl, pH7.5, 150mM NaCl, 1.5mMgCl2, 0.5% NP-40) containing protease inhibitors. Lysates were precleared with Protein G agarose for 1 hour at 4°C and then incubated with a GFP antibody (Roche 11814460001) for 1 hour at 4°C. Protein G agarose was added and incubated for an additional 3 hours to precipitate the immunocomplexes. The agarose beads were washed 4 times in lysis buffer, boiled in 2X SDS sample buffer, and analyzed by Western blot with indicated antibodies. For DNA-independent co-immunoprecipitations, ethidium bromide at indicated concentrations or 4μl of DNase (Roche) was added during the second wash step and incubated for 30 minutes at 4°C. For co-immunoprecipitations from *sos-1* mutants, synchronized L2 animals were shifted from 20°C to 25°C. Adult worms were harvested the next day for co-immunoprecipitation as described above.

**GST pulldowns:** GST (pGEX-4T1) and GST::EOR-1 (pPM1) were purified from BL21-Gold (DE3) cells (Stratagene) using glutathione sepharose 4B beads (Amersham Pharmacia). Beads were washed in buffer (50mM Tris-HCl, pH7.5, 150mM NaCl, 1.5mMgCl2, 0.5% NP-40) containing protease inhibitors and incubated for 3.5 hours at 4°C with Gal4DBD- or 3xFLAG-fusion proteins expressed using the TNT T7 Quick Coupled Transcription/Translation System.
(Promega). Beads were washed 4 times in buffer, boiled in 2X SDS sample buffer, and analyzed
by Western blot with FLAG and GAL4DBD antibodies described above.

**Transgenic lines and rescue experiments:** For expression of *eor-1* and *eor-2* under the
control of their own promoter, transgenic lines were made by injecting pRH5 (*eor-1p::EOR-
1::GFP*), pKH47 (*eor-1p::EOR-1(L81F)::GFP*), pKH53 (*eor-1p::EOR-1S64A::GFP*), pKH54
(*eor-1p::EOR-1S248A::GFP*), pKH55 (*eor-1p::EOR-1S64A:S248A::GFP*), or pKH56 (*eor-
1p::EOR-1S64A:S87A:S248A::GFP*), pRH29 (*eor-2p::EOR-2::GFP*), or pKH52 (*eor-2p::EOR-
2(C900S)::GFP*) at 15 ng/µℓ with *ttx-3p::GFP* (described previously, ALTUN-GULTEKIN et al.
2001) at 30ng/µℓ, and pBluescript SK+ (Stratagene) at 55ng/µℓ. For tissue-specific expression in
the hypodermis, transgenic lines were made by injecting pKH14 (*dpy-7p::EOR-1*) or pKH15
(*dpy-7p::EOR-2*) at 10ng/µℓ with *pTG96_2* (*sur-5::GFP*, described previously, GU et al.
1998) at 30ng/µℓ, and pBluescript SK+ at 60ng/µℓ. For tissue-specific expression in the VPCs,
transgenic lines were made by injecting pKH24 (*lin-31p::EOR-1*) or pKH25 (*lin-31p::EOR-2*) at
100ng/µℓ with *pTG96_2* at 30ng/µℓ. *lin-39(n709); eor-1(cs28)* and *lin-39(n709); eor-2(cs30)*
animals carrying these transgenes were scored at the L4 stage for wild-type division of P6.p.
Siblings lacking the transgenes were scored as controls.

**ERK in vitro kinase assays:** GST::EOR-1 (from pPM1), GST::EOR-1(L81F) (from
pKH51), GST::EOR-1N (from pKH49), and GST::EOR-1C (from pKH50) were expressed in
BL21(DE3) cells (Sigma). Kinase assays with purified protein and murine ERK2 (NEB P6080L)
were performed as previously described (ARUR et al. 2009).
RESULTS

EOR-1(L81F) is a putative null allele but does not affect EOR-1 expression or localization: To better understand the role of the EOR-1 BTB domain, we characterized cs44, an allele of eor-1 that encodes for the point mutation L81F that is located in this domain (HOWARD and SUNDARAM 2002); shown in Fig. 1A,B,C). The phenotypes of eor-1(cs44) animals are similar to those of the molecular null eor-1(cs28), suggesting cs44 is also a strong loss of function allele (ROCHELEAU et al. 2002).

We generated a polyclonal peptide antibody against EOR-1 (described in Materials and Methods) and tested if L81F affected the expression of EOR-1 in cs44 animals. In Western analyses, this antibody recognizes a band at the predicted size of EOR-1 (~115kDa) in wild-type animals that is absent in eor-1(cs28)-null animals (Fig. 1D). This band is present in eor-1(cs44) animals at an amount similar to that in the wild-type strain; therefore, L81F does not affect the protein levels of EOR-1 in vivo.

We then tested whether L81F affects localization of EOR-1 in vivo. A functional eor-1p::EOR-1::GFP reporter transgene capable of rescuing eor-1 mutant defects is expressed in the nuclei of most cells throughout development, including the VPCs (HOWARD and SUNDARAM 2002 and Fig. 1E). An equivalent eor-1p::EOR-1(L81F)::GFP transgene also showed nuclear-localized expression of EOR-1(L81F)::GFP in most cells throughout development. This indicates that L81F also does not disrupt the localization of EOR-1.

Finally, we tested if L81F has any detectable activity when overexpressed in a transgenic rescue assay. Our assay is based on the previously described role of eor-1 in Ras/ERK-mediated vulval fate induction (HOWARD and SUNDARAM 2002). There are six vulval precursor cells (VPCs) in the worm that have the ability to adopt one of three cell fates: 1°, 2°, and 3° (reviewed
in STERNBERG 2005). These fates are characterized by the number of divisions the cell undergoes (see Fig. 2A). In wild-type, the VPC P6.p adopts a 1° vulval fate and divides three times. In let-60 ras or lin-39 Hox mutants, P6.p adopts a 3° fate and divides only once (or not at all), and the animals are Vulvaless (Vul). Although eor-1 single mutants have wild-type vulva development, in appropriate sensitized genetic backgrounds such as lin-39(n709) (a weak hypomorphic allele), eor-1 mutations can prevent P6.p from adopting the 1° fate and thus cause a Vul phenotype (HOWARD and SUNDARAM 2002). We found that while the wild-type eor-1p::EOR-1::GFP transgene efficiently rescued the lin-39; eor-1 Vul phenotype, eor-1p::EOR-1(L81F)::GFP showed no rescue activity (Fig. 2B). Furthermore, eor-1p::EOR-1(L81F)::GFP did not cause vulval defects in the lin-39 single mutant background and thus does not behave like a dominant-negative (Fig. 2B). We conclude that, despite its normal expression and localization, L81F eliminates all tested eor-1 functions.

**EOR-1(L81F) affects a predicted interaction surface:** To understand how L81F might eliminate EOR-1 function, we wanted to determine where the mutation might lie in the secondary structure of the BTB domain. The crystal structures of BTB domains from several BTB-zinc finger proteins such as PLZF and BCL-6 have been solved (AHMAD et al. 1998; AHMAD et al. 2003) and show striking overlap, suggesting that most BTB domains will adopt a similar structure. The secondary structures of these BTB domains consist of a core of 6 α-helices surrounded by 5 β-sheets at the top and bottom (depicted in Fig. 1B). Both PLZF and BCL-6 BTB domains form obligate homodimers mediated by interactions between β1, α1, A1, A2, and β5. Dimerization of the BTB domain creates two surfaces important for interactions with other proteins: the lateral groove located at the bottom of the dimer and the charged pocket located at
the top of the dimer (Ahmad et al. 2003; Ghetu et al. 2008; Melnick et al. 2002). The lateral groove in the BCL-6 BTB dimer structure has been shown through co-crystallization studies to make contacts with co-repressor proteins SMRT (Ahmad et al. 2003) and BCOR (Ghetu et al. 2008). In addition, mutation of conserved charged residues within the charged pocket disrupts the ability of PLZF and BCL-6 to bind the N-Cor and SMRT corepressors (Melnick et al. 2000; Melnick et al. 2002), implicating the charged pocket in cofactor binding.

Using ClustalW, we aligned the sequences of EOR-1 from C. elegans and the related nematode species C. briggsae with human PLZF and human BCL-6, and then compared this alignment to the known secondary structures (Fig. 1C). Interestingly, L81F is predicted to fall in B3, an exposed β-sheet that lies at the top of the monomer and near the opening of the charged pocket of the dimer (Fig. 1B and 1C). This same β-sheet in the BTB-zinc finger protein MIZ-1 is necessary for the protein’s tetramerization (Stead et al. 2007); similarly, in the BTB domain of the ubiquitin ligase complex component Skp1, this β-sheet makes contacts with the protein’s binding partner, Cul1 (Zheng et al. 2002). Based on its predicted location on an exposed surface, we hypothesized that L81F could affect the interaction of EOR-1 with other proteins.

EOR-1(L81F) disrupts an interaction with EOR-2: To test whether L81F disrupts the binding of EOR-1 to other proteins, we co-transfected Gal4DBD::EOR-1 and various 3xFLAG-fusion proteins into 293T cells for co-immunoprecipitation experiments. 293T lysates were immunoprecipitated with a Gal4DBD antibody and immunocomplexes were analyzed by Western blot using a FLAG antibody.

We first asked if EOR-1 had the ability to interact with itself as most other BTB-zinc finger proteins form dimers. 3xFLAG::EOR-1 co-immunoprecipitated with Gal4DBD::EOR-1,
suggesting EOR-1 can dimerize (Fig. 3A). L81F did not disrupt this interaction since 3xFLAG::EOR-1 could still co-immunoprecipitate with Gal4DBD::EOR-1(L81F). Furthermore, EOR-1(L81F) can also interact with itself as 3xFLAG::EOR-1(L81F) co-immunoprecipitated with Gal4DBD::EOR-1(L81F) (Fig. 3B). Thus, consistent with its predicted location away from the dimer interface, L81F does not prevent EOR-1 dimerization.

We next tested if EOR-1 could bind to EOR-2, since genetic evidence suggests EOR-1 and EOR-2 function together in vivo. Indeed, 3xFLAG::EOR-2 co-immunoprecipitated with Gal4DBD::EOR-1 from 293T cells (Fig. 3C). Interestingly, however, this interaction was completely disrupted by L81F; little or no 3xFLAG::EOR-2 co-purified with Gal4DBD::EOR-1(L81F), despite the fact that GAL4DBD::EOR-1(L81F) was immunoprecipitated at levels similar to wild-type Gal4DBD::EOR-1. These results suggest that L81F disrupts the ability of EOR-1 to bind EOR-2, and that formation of an EOR-1-EOR-2 complex may be critical for biological function.

We further mapped the interaction between EOR-1 and EOR-2 by expressing deletion constructs of the proteins in the 293T system. For the EOR-1 deletion constructs, Gal4DBD::EOR-1N contains the protein’s BTB and BACK domain, whereas Gal4DBD::EOR-1C contains the protein’s zinc finger domains and polyglutamine stretch (Fig. 3F). Gal4DBD::EOR-1N was both necessary and sufficient for the protein’s interaction with itself (3xFLAG::EOR-1) and with 3xFLAG::EOR-2 (Fig. 3D). Gal4DBD::EOR-1C did not interact with either 3xFLAG::EOR-1 or 3xFLAG::EOR-2. We also tested the ability of EOR-2 to interact with PLZF, a related mammalian BTB-zinc finger protein that lacks a BACK domain. 3xFLAG::EOR-2 was also able to co-immunoprecipitate with GAL4DBD::PLZF from 293T cells (Fig. 3E). These mapping experiments are consistent with the idea that the BTB domain is a
major interaction domain of EOR-1, and also raise the possibility that PLZF or other BTB-zinc finger proteins in other organisms may utilize an EOR-2-like partner (see Discussion).

The conserved CHHC motif in EOR-2 is important for EOR-1 binding and in vivo function: EOR-2 does not contain any recognized functional motifs but its C-terminus shares weak similarity with uncharacterized proteins from other organisms, including Drosophila and humans (Hoeppner et al. 2004 and Fig. 4A). Within this weakly conserved region is a CHHC zinc finger-like motif, albeit with atypical spacing.

To map regions of EOR-2 important for interaction with EOR-1, we made deletion constructs separating the region of weak conservation between EOR-2-related proteins (3xFLAG::EOR-2C) from the rest of the protein (3xFLAG::EOR-2N) (Fig. 4D). Neither the N or C terminal deletion constructs by themselves were sufficient for the interaction with EOR-1 (Fig. 4B). Given that some zinc finger domains have roles in protein-protein interactions (reviewed in Gamsjaeger et al. 2007), we asked whether disrupting the CHHC zinc finger-like domain of EOR-2 would affect binding to EOR-1. We mutated the first cysteine (C900) in this motif to a serine (Fig. 4A) and asked if the EOR-2(C900S) protein could still interact with EOR-1. Despite being expressed at normal levels, 3xFLAG::EOR-2(C900S) showed a significantly reduced ability to co-immunoprecipitate with Gal4DBD::EOR-1 (Fig. 4C). This result suggests that the conserved zinc finger-like motif of EOR-2 is important for its interaction with EOR-1.

To test the biological function of EOR-2(C900S), we turned to our in vivo lin-39; eor-2 vulval rescue assay (Fig. 2). While we saw significant rescue of the P6.p division defect with the wild-type eor-2p::EOR-2::GFP transgene, none of the 3 lines carrying an equivalent eor-2p::EOR-2(C900S)::GFP transgene rescued the Vul phenotype of the lin-39; eor-2 animals.
Importantly, non-functional EOR-2(C900S)::GFP was still detectably expressed and localized to VPC nuclei (Fig. 4E). Taken together, the EOR-1(L81F) and EOR-2(C900S) results suggest that the ability of EOR-1 and EOR-2 to interact is critical for both of the proteins’ function.

**EOR-1 and EOR-2 interact in vivo:** Having shown that EOR-1 and EOR-2 can physically interact in cell culture, we next asked whether the two proteins interact *in vivo*. We first tested whether EOR-1 and EOR-2 act in the same cells as each other by expressing the proteins under the control of tissue-specific promoters during vulva development and looking for rescue of *lin-39; eor* mutant phenotypes (Fig. 2B), as described above. To test whether the EOR-1 and EOR-2 function in the VPCs where Ras is active, we expressed the proteins under the control of the vulval *lin-31* promoter (Tan *et al.* 1998). As a control, we also put EOR-1 and EOR-2 under the control of the *dpy-7* promoter (Gilleard *et al.* 1997; Myers and Greenwald 2005) to drive expression in the surrounding hypodermis. Several lines were generated for each transgene in the *lin-39; eor* background. We saw significant rescue of the P6.p division defects when EOR-1 or EOR-2 were expressed in the VPCs (Fig. 2B). However, the vulva phenotypes were not significantly rescued when EOR-1 or EOR-2 were expressed in the surrounding hyp7. These results indicate that EOR-1 and EOR-2 function together in the VPCs and are consistent with the epistasis experiments that place EOR-1 and EOR-2 acting downstream of ERK.

We then asked whether the proteins physically interact *in vivo*. We expressed a rescuing transgene of EOR-2 fused to GFP (Howard and Sundaram 2002 and Fig. 2B) along with the co-injection marker *ttx-3p::GFP* in *eor-2* mutants. We immunoprecipitated EOR-2::GFP with a GFP antibody and analyzed proteins that were co-purified by immunoblotting with the EOR-1 antibody described above. EOR-1 was present in EOR-2::GFP immunoprecipitates from staged
L4 animals (Fig. 5A), as well as from adults (data not shown) and mixed stage animals (Fig. 5B and 5C). This interaction was specific, because EOR-1 was not detectable in GFP immunoprecipitates from eor-1(cs28) null; EOR-2::GFP animals or from animals expressing only the co-injection marker ttx-3p::GFP. Furthermore, we did not detect the control protein HSP90 or another BTB domain protein, MEL-26, in EOR-2::GFP immunoprecipitates (Fig. 5B). To ask if EOR-1(L81F) could interact with EOR-2 in vivo, we crossed the EOR-2::GFP transgene into eor-1(cs44) animals which express endogenous EOR-1(L81F). EOR-2::GFP did not immunoprecipitate EOR-1(L81F) from staged L4 eor-1(cs44) animals. These results suggest that EOR-1 and EOR-2 form a complex in vivo, and consistent with our 293T results, the L81F mutation in EOR-1 disrupts this complex formation.

Since EOR-1 and EOR-2 both have zinc finger domains that may bind DNA, we asked whether the interaction between the two proteins is DNA-dependent. EOR-2::GFP was immunoprecipitated from eor-2 mutant animals as described above in the presence of ethidium bromide and DNase to disrupt DNA. EOR-1 still associated with EOR-2::GFP in these conditions, demonstrating that the interaction between EOR-1 and EOR-2 is DNA-independent (Fig. 5C).

As discussed in the Introduction, other BTB-zinc finger proteins have been shown to interact with histone deacetylase (HDAC) complexes. To test if this is also the case for EOR-1, we probed EOR-1::GFP immunoprecipitates with an antibody against the C. elegans class I HDAC, HDA-1 (Fig. 5D). HDA-1 did not co-immunoprecipitate with EOR-1::GFP from worm lysates, indicating that EOR-1 may not function with HDA-1 in vivo.
**EOR-1(L81F) disrupts a putative ERK docking site:** We noticed that L81F also disrupts a potential ERK docking site in EOR-1. ERK uses docking sites on its substrates to recognize and subsequently phosphorylate those substrates at specific phosphoacceptor sites (S/TP). Several docking sites have been described for ERK, including the D-Domain, which is defined by several basic residues followed by a L/I-X-L/I motif (Jacobs et al. 1999; Yang et al. 1998a; Yang et al. 1998b). EOR-1 is a candidate ERK target based on genetic arguments (Introduction), and EOR-1 contains two putative D-Domains: one in the BTB domain and one in the BACK domain (Fig. 1A). The leucine mutated in L81F corresponds to the first leucine in the L-X-I motif of the D-Domain in the BTB domain of EOR-1 (Fig. 1C).

We performed *in vitro* kinase assays to determine if EOR-1 is phosphorylated by active ERK and to test if L81F affects the affinity of the kinase for EOR-1 as a substrate (Fig. 6A). Kinetic analysis was performed from these assays to measure the Km and Vmax, which were used to calculate the protein’s relative acceptor ratio (RAR, defined as Vmax/Km) (Fig. 6B). The RAR is the overall measure of a protein as a substrate (Jacobs et al. 1998). Myelin basic protein (MBP) was used to normalize the RAR values in these assays because it contains several phosphoacceptor sites but lacks any known ERK docking sites. LIN-1, an ETS family transcription factor and known target of *C. elegans* MPK-1/ERK, was used as a positive control (Jacobs et al. 1998). In these kinase assays, GST::EOR-1 had an RAR value higher than LIN-1, indicating it is also a robust substrate of ERK *in vitro*. GST::EOR-1(N) (Fig. 3F), which contains the BTB and BACK domains, both D-domains, and three potential phosphoacceptors, also had a high RAR value, consistent with phosphorylation occurring in this region (Fig. 6C, D). However, GST::EOR-1(L81F) had a much lower RAR value; this decrease is mostly due to a 4-fold higher Km. The higher Km of EOR-1(L81F) suggests that ERK has a reduced affinity for
the mutant protein. These results are consistent with the idea that L81F reduces the ability of EOR-1 to interact with and be phosphorylated by ERK, and raise the possibility that phosphorylation promotes EOR-1 function.

The interaction between EOR-1 and EOR-2 is direct and may occur independently of Ras signaling: Since L81F reduces the ability of EOR-1 to serve as an ERK substrate and also disrupts binding to EOR-2, we considered the possibility that ERK phosphorylation promotes EOR-1-EOR-2 binding. However, several observations discussed below argue against a strict version of this model.

First, reducing Ras signaling \textit{in vivo} did not perturb the EOR-1-EOR-2 interaction. We used a temperature sensitive allele of \textit{sos-1}, the guanine exchange factor for the Ras GTPase (described in Rocheleau \textit{et al.} 2002) to decrease Ras/Erk signaling \textit{in vivo}. When shifted to the restrictive temperature at the L2 stage, \textit{sos-1(ts);eor-2; EOR-2::GFP} animals displayed a strong Vul phenotype, indicating that Ras signaling is severely compromised; however, under these conditions, EOR-1 still co-immunoprecipitated with EOR-2::GFP at levels similar to those seen previously (Fig. 7A).

Second, EOR-1 and EOR-2 can interact \textit{in vitro}. GST::EOR-1 was purified from bacteria with glutathione beads and incubated with \textit{in vitro} transcribed and translated 3xFLAG::EOR-2. As shown in Fig. 7B, 3xFLAG::EOR-2 was pulled down with GST::EOR-1 but not GST alone. For controls, we tested whether GAL4DBD::EOR-1N and GAL4DBD::EOR-1C were pulled down with GST::EOR-1. Similar to the 293T co-immunoprecipitation experiments, only GAL4DBD::EOR-1N was pulled down with GST::EOR-1, indicating these interactions are
specific. These results suggest that the interaction between EOR-1 and EOR-2 is direct and does not require other bridging proteins or modifications such as phosphorylation.

Third, mutation of the three potential phosphoacceptor sites in the EOR-1 N-terminus (S64, S87 and S248) reduced activity in our *in vivo* transgenic rescue assays, but unlike L81F, did not eliminate activity (Fig. 2). While these results are consistent with the proposal that phosphorylation promotes EOR-1 function, it appears that loss of N-terminal phosphorylation has less severe consequences than loss of EOR-2 binding.

Together with the observation that EOR-1 and EOR-2 function together to promote some processes that do not appear to involve Ras/ERK signaling (Hoeppner *et al.* 2004), these results suggest that the interaction between EOR-1 and EOR-2 is most likely constitutive and independent of Ras/ERK signaling.

**DISCUSSION**

We have shown that the novel protein EOR-2 is an obligate binding partner of the BTB-zinc finger protein EOR-1 in *C. elegans*. EOR-1 and EOR-2 appear to interact constitutively throughout development, and their interaction correlates strictly with biological function. An endogenous missense mutation (L81F) in the EOR-1 BTB domain eliminates the EOR-2 interaction and all detectable biological function without affecting EOR-1 stability, localization, or dimerization. Our analysis suggests that the interaction between EOR-1 and EOR-2 may be mediated by the charged pocket region of the EOR-1 BTB dimer, a region that, in other BTB-zinc finger proteins, has been proposed to interact with corepressors or coactivators. We propose that EOR-2 defines a new family of cofactors for BTB-zinc finger transcription factors that may have conserved roles in other organisms.
**EOR-2 is a dedicated binding partner of EOR-1:** Our discovery of an EOR-1-EOR-2 protein complex is consistent with prior genetic studies suggesting that these two proteins function closely together to promote Ras/ERK-dependent cell fates, sex-specific cell death programs, and other developmental processes in *C. elegans* (HOEPPNER et al. 2004; HOWARD and SUNDARAM 2002). The interaction between EOR-1 and EOR-2 is direct and maps to the BTB domain of EOR-1. Although *C. elegans* has only one other BTB-zinc finger protein besides EOR-1 (and its function is uncharacterized), the worm has many other BTB-containing proteins, including a particularly large class of BTB-MATH proteins (STOGIOS et al. 2005). Nevertheless, there is no evidence to suggest that EOR-2 functions with any of these other BTB-containing proteins. First, both eor-1 and eor-2 have been consistently pulled out together from genetic screens in which no other BTB-domain proteins were identified (HOEPPNER et al. 2004; HOWARD and SUNDARAM 2002). In addition, the phenotypes of eor-2 mutants appear identical to those of eor-1 mutants, and lethal defects described for other BTB-domain protein mutants are not observed in eor-2 mutants (DOW and MAINS 1998; OHMACHI et al. 1999). Furthermore, we did not detect an interaction between EOR-2 and the BTB-MATH protein MEL-26 *in vivo*. Therefore, EOR-2 appears to function as a dedicated binding partner of EOR-1.

**EOR-2-related proteins may share a conserved role as binding partners of BTB-zinc finger proteins:** EOR-2-related proteins are also found in other organisms (HOEPPNER et al. 2004), but their functions have yet to be characterized. Conservation among these proteins is limited to a C-terminal region that includes a CHHC zinc finger-like motif. We showed that this CHHC motif is essential for EOR-2 function *in vivo* and for binding to EOR-1. Notably, EOR-2
was able to interact with the human BTB-zinc finger protein PLZF when both proteins were over-expressed in 293T cells. This raises the possibility that EOR-2-related proteins may bind and function with BTB-zinc finger proteins in other organisms.

Several factors might explain why EOR-2-related proteins have not previously been identified as BTB-zinc finger partners. First, in contrast to *C. elegans*, which has only two BTB-zinc finger proteins, *Drosophila* and mammals have many members of this protein family (STOGIOS et al. 2005). If EOR-2 related proteins function with multiple BTB-zinc finger family members, then their mutant phenotypes may be very pleiotropic, precluding their isolation through most standard genetic approaches. Second, EOR-2-related proteins may not be amenable to discovery through yeast two-hybrid screens or other tag-based biochemical approaches. We found that GAL4::EOR-2 fusion proteins failed to interact with EOR-1 in yeast two-hybrid assays, and GST::EOR-2 failed to interact with EOR-1 *in vitro* (data not shown). It appears that attachment of a bulky tag to the EOR-2 N-terminus interferes with its ability to bind EOR-1. Only EOR-2 proteins fused to smaller tags (such as 3X FLAG) or tagged at their C-termini were functional and able to bind EOR-1. Our discovery that EOR-2 is an important EOR-1 binding partner should now motivate direct tests of EOR-2-related proteins in other systems.

The EOR-1-EOR-2 complex may promote Ras/ERK-dependent gene expression:

Our interest in EOR-1 and EOR-2 originally stemmed from their role as downstream mediators of Ras/ERK signaling (HOWARD and SUNDARAM 2002). One very important role of the Ras/ERK pathway in *C. elegans* is to upregulate expression of Hox genes such as *lin-39* (in the vulva) (EISENMANN et al. 1998; MALOOF and KENYON 1998; WAGMAISTER et al. 2006a; WAGMAISTER
et al. 2006b) and egl-5 (in the P12 ectoblast) (Jiang and Sterner Berg 1998; Li et al. 2007). The phenotypes and genetic properties of eor-1 and eor-2 mutants are consistent with the model that the EOR-1-EOR-2 complex also functions to promote expression of these Hox genes (Howard and Sundaram 2002), though whether it does so directly or indirectly is unknown.

If EOR-1 and EOR-2 directly promote Hox expression, then that would imply that they function as transcriptional activators. Other BTB-zinc finger proteins can function as repressors or activators (or both), and, interestingly, several also regulate Hox gene expression: PLZF represses HoxD and Hoxb2 in the mouse (Baran et al. 2002; Ivins et al. 2003), whereas GAGA maintains expression of several Hox genes in Drosophila (Shimomura et al. 2003). Although EOR-1 is most similar to PLZF in overall identity and in numbers and spacing of the C2H2 domains, PLZF contains an additional repressor domain (RD2) that EOR-1 lacks (Li et al. 1997a). In addition, so far we have been unable to find evidence that EOR-1, like PLZF, interacts with class I histone deacetylases (HDACs). Furthermore, unlike PLZF but similar to GAGA, EOR-1 has a polyglutamine domain at its C-terminus. The polyglutamine domain in GAGA has been implicated in transcriptional activation (Vaquero et al. 2008; Vaquero et al. 2000). Therefore it is quite possible that the EOR-1-EOR-2 complex functions to activate transcription of target genes. Few BTB-domain binding partners have been identified for BTB-zinc finger proteins that function as transcriptional activators. EOR-2 could be such a partner.

What might be the relevant function of EOR-2 in the EOR-1-EOR-2 complex? Our data and those of Hoeppner et al. (2004) and Howard and Sundaram (2002) indicate that EOR-2 is not required for the stability or for the proper localization of EOR-1. One possibility is that EOR-2 binding could allow EOR-1 to interact with DNA or other proteins. EOR-2 contains a zinc finger-like domain, so EOR-2 may help EOR-1 associate with specific target gene
promoters by binding to DNA sequences itself. If EOR-1 were unable to bind DNA in the absence of EOR-2, this might explain why EOR-1(L81F) does not behave as a dominant-negative, even when overexpressed. Alternatively, EOR-2 could act as a bridging partner between EOR-1 and TFIID components or other co-activators or co-repressors critical for its function. Conversely, EOR-2 could block EOR-1 from binding a protein that inhibits its activity. Finally, EOR-2 may carry uncharacterized domains with activator or repressor activity necessary for the EOR complex to regulate gene expression.

**EOR-1 may be a direct MPK-1/ERK target:** Our genetic and *in vitro* phosphorylation studies suggest that EOR-1 may be a direct target of MPK-1/ERK *in vivo*, but how phosphorylation affects EOR-1 is not yet clear. Although the EOR-1(L81F) point mutation disrupts both the ability of EOR-1 to be phosphorylated by ERK and to bind EOR-2, our results do not support a model in which the phosphorylation of EOR-1 is absolutely required for its interaction with EOR-2. We found that EOR-1 and EOR-2 can bind *in vitro* in the absence of ERK phosphorylation and *in vivo* even when Ras signaling is diminished. Furthermore, mutation of the three potential phosphoacceptor sites in the EOR-1 N-terminus reduces but does not abolish activity in transgenic rescue assays, suggesting that some EOR-2 binding is retained. Although not necessary for binding to EOR-2, phosphorylation of EOR-1 might contribute to the stability of the EOR-1-EOR-2 complex *in vivo* or might promote some other aspect of complex activity. Further studies of specific phosphoacceptor site mutants, along with identification of EOR-1-EOR-2 target genes, will be needed to test these possibilities.

Interestingly, several BTB-zinc finger proteins have been shown to be negatively regulated by Ras/ERK signaling. During B-cell development, ERK directly phosphorylates BCL-
6, targeting it for degradation through the ubiquitin/proteosome pathway (Moriyama et al. 1997; Ni et al. 1998). In Drosophila, Ras/ERK signaling indirectly triggers degradation of the BTB-zinc finger protein Tramtrack by modulating the activity of the E3 ubiquitin ligase Sina (Li et al. 1997b; Li et al. 2002). In a recent study of myeloid development, activation of ERK was shown to induce the export of PLZF from the nucleus (Doulatov et al. 2009). In contrast to these examples of negative regulation, our data suggest that ERK phosphorylation of EOR-1 might positively influence EOR-1 activity.

In summary, we have shown that EOR-1 and EOR-2 form a functional complex in vivo and propose that the Ras/ERK pathway regulates the activity of this complex to promote Ras-dependent cell fates. Further studies of the EOR-1-EOR-2 complex should provide insight into how Ras/Erk signaling controls developmental processes and how BTB-zinc finger proteins regulate transcription of target genes.

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FIGURE LEGENDS

Figure 1. EOR-1(L81F) lies in a potential interaction surface of the BTB domain and does not affect protein expression or localization

(A) EOR-1 protein schematic. EOR-1 contains a BTB domain, a BACK domain, 9 zinc finger domains, and a polyglutamine stretch (Q). L81F falls in BTB domain of EOR-1. EOR-1 contains 2 potential ERK (D-Domain) docking sites (K/R-X-X-K/R-X(1-4)-L/I-X-L/I) (JACOBS et al. 1999; YANG et al. 1998a; YANG et al. 1998b), and 10 potential SP or TP phosphoacceptor sites (asterisks).

(B) Ribbon diagram of the PLZF BTB domain dimer (modified from AHMAD et al. 1998) with secondary structures nomenclature from (STOGIOS et al. 2005)). Predicted location of EOR-1(L81F) and potential ERK phosphoacceptor sites in each monomer are indicated with arrows and asterisks, respectively.

(C) ClustalW alignment of BTB domains of *C. elegans* EOR-1, *C. briggsae* EOR-1, human PLZF, and human BCL-6. Amino acids predicted to be in α-helices and β-sheets based on PLZF crystal structure are underlined (AHMAD et al. 1998; AHMAD et al. 2003). Identical residues are highlighted. Conserved residues are boxed. Arrow marks the L81F point mutation in EOR-1.
Potential D-Domain ERK docking site is highlighted in red. Asterisks mark potential ERK phosphoacceptor sites. Note that the D-domain is conserved in C. briggsae EOR-1. In addition, EOR-1, PLZF and BCL-6 all contain D-domains between the BTB and zinc finger regions (not shown).

(D) L81F mutation does not affect EOR-1 levels. Protein lysates from wild-type (WT), eor-1 (cs28) null, and eor-1 (cs44) L81F staged L4 animals were subjected to Western blotting and probed with antibodies for EOR-1 and HSP90 (loading control).

(E) L81F mutation does not affect localization of EOR-1. EOR-1::GFP (top) and EOR-1L81F::GFP (bottom) are expressed in the nuclei of many cells, including the VPCs, during the L3 stage. Arrows indicate cells P8.p (left/posterior) to P5.p (right/anterior).

Figure 2. EOR-1 and EOR-2 function in the VPCs and point mutants that disrupt the interaction between the two proteins disrupt their function.

(A) Model for vulva development (reviewed in STERNBERG 2005). The EGF-like ligand LIN-3 is released from the anchor cell (AC) and activates the Ras/ERK signaling cascade in P6.p to specify the 1° cell fate. In addition to receiving some LIN-3 signaling, P5.p and P7.p also receive a lateral signal from P6.p via Notch, causing them to adopt the 2° cell fate. P3.p, P4.p, and P8.p, which do not receive any signal, divide once, and fuse to the surrounding hyp7 tissue (3° cell fate). The wild-type lineage of each VPC is depicted below each cell.

(B) eor-1 and eor-2 were expressed under the control of either endogenous or tissue-specific promoters (lin-31p (VPCs) and dpy-7p (hyp7)) in lin-39(n709); eor-1(cs28); lin-39(n709) or lin-39(n709); eor-2(cs30) mutant backgrounds. L4 animals were scored for wild-type P6.p division.

a(HOWARD and SUNDARAM 2002); **p<0.01, Fisher’s exact test, compared to siblings not
carrying the transgene;* 0.01<p<0.05, Fisher’s exact test, compared to siblings not carrying the transgene.

**Figure 3. L81F disrupts EOR-1-EOR-2 interaction but not EOR-1 multimerization.**

(A-E) 293T cells were transfected with Gal4DBD (GAL4)- and 3xFLAG (FL)-fusion constructs (indicated at top of each lane). Lysates were immunoprecipitated with a Gal4DBD antibody and purified immunocomplexes were analyzed by Western blot using FLAG and Gal4DBD antibodies. In all experiments, 3xFLAG::EBNA1 was used as a negative control and did not co-immunoprecipitate with Gal4DBD fusion proteins. Several of the GAL4DBD-fusion proteins were detected as two bands in the size range expected for the constructs by the GAL4DBD antibody. In several panels, 3xFLAG::EOR-1 expression is barely detectable in the input lanes; despite this low expression, however, 3xFLAG::EOR-1 is still able to be co-immunoprecipitated at a high level. Some differences in the amount of protein co-immunoprecipitated were observed between experiment (for example, compare amount of 3xFLAG::EOR-1 co-immunoprecipitated with GAL4DBD::EOR-1 in Fig. 3A and Fig. 3B), but this is most likely due to variation in the expression levels of proteins and in the efficiency of the immunoprecipitations between experiments. Co-immunoprecipitations in panels A and C were performed at least 4 times whereas co-immunoprecipitations in panels B, D, and E were performed at least 2 times.

(A) EOR-1 self-interacts and L81F does not disrupt this interaction.

(B) EOR-1(L81F) self-interacts.

(C) EOR-1 and EOR-2 interact and this binding is disrupted by L81F.

(D) EOR-1N is necessary and sufficient for the interaction of EOR-1 with itself and with EOR-2.

(E) EOR-2 can interact with PLZF.
Summary of EOR-1 deletion constructs and interactions in 293T cells. Asterisk marks L81F mutation in EOR-1. PLZF was also used in 293T experiments and contains a BTB domain, RD2 repression domain and 9 zinc finger domains.

Figure 4. The conserved CHHC zinc finger of EOR-2 is necessary for the interaction with EOR-1.

(A) Alignment of the C termini of C. elegans EOR-2, C. briggsae EOR-2, Drosophila CG17233, and human NP_079001.2 (modified from Hœppner et al. 2004). Identical residues are highlighted. Conserved residues are boxed. CHHC zinc finger-like domain amino acids are in red. Asterisk marks the cysteine mutated in EOR-2C900S.

(B-C) Co-immunoprecipitations were performed 2 times as described in Figure 3.

(B) Neither EOR-2N nor EOR-2C is sufficient for the interaction with EOR-1.

(C) Disruption of CHHC zinc finger-like domain of EOR-2 disrupts its interaction with EOR-1.

(D) Summary of EOR-2 deletion constructs and interactions in 293T cells. Black indicates the conserved region shown in A and gray indicates the CHHC zinc finger-like motif. Asterisk marks C900S mutations in EOR-2.

(E) EOR-2C900S::GFP is expressed in the nuclei of VPCs at the L3 stage. Arrowheads indicate P6.p (left/posterior) to P4.p (right/anterior).

Figure 5. EOR-1 and EOR-2 specifically interact in vivo and DNA is not required for this interaction.

(A-D) Lysates from animals carrying either eor-1p::EOR-1::GFP; ttx-3p::GFP (EOR-1::GFP), eor-2p::EOR-2::GFP; ttx-3p::GFP (EOR-2::GFP), or the co-injection marker ttx-3p::GFP alone
were immunoprecipitated with a GFP antibody and immunocomplexes were analyzed by Western blot with various antibodies. *eor-1(cs28)* or *eor-2(cs30)* null backgrounds (Howard and Sundaram 2002) were used to eliminate endogenous EOR protein expression.

(A) EOR-1 and EOR-2 interact *in vivo* and the interaction is disrupted in EOR-1(L81F) animals. EOR-1 co-immunoprecipitated with EOR-2::GFP from staged *eor-2 L4* animal extracts. EOR-1(L81F) did not co-immunoprecipitate with EOR-2::GFP from *eor-1(cs44); eor-2* staged L4s. EOR-1 was not present and therefore did not co-immunoprecipitate with EOR-2::GFP in *eor-1(cs28)* null animals. EOR-1 did not co-immunoprecipitate from *eor-2* animals only expressing the co-injection marker *ttx-3p::GFP*. HSP90 was used to demonstrate equal loading and did not co-immunoprecipitate in any experiment.

(B) EOR-2 does not interact with the BTB-MATH protein MEL-26 *in vivo*. EOR-1 but not MEL-26 co-immunoprecipitated with EOR-2::GFP from mixed stage *eor-2* animal extracts. A non-specific band at a molecular weight higher than MEL-26 was detected in control immunoprecipitates from *ttx-3p::GFP* animals (Asterisk indicates non-specific band; arrow indicates where MEL-26 should migrate).

(C) The interaction between EOR-1 and EOR-2 does not require DNA. EOR-1 still co-immunoprecipitates with EOR-2::GFP from mixed stage *eor-2* animals in the presence of DNase and of varying concentrations of ethidium bromide (EtBr).

(D) EOR-1 does not interact with HDA-1 *in vivo*. HDA-1 did not co-immunoprecipitate with EOR-1::GFP from mixed stage *eor-1(cs28)* null animals.

Figure 6. EOR-1 is robustly phosphorylated by ERK *in vitro* and L81F disrupts this phosphorylation.
(A) Graph of counts per minute (CPM) versus protein concentration (nanomolar) for GST-tagged EOR-1(WT) (blue line), GST-tagged EOR-1(L81F) (black line), GST-tagged LIN-1 as positive control (orange line) or myelin basic protein (MBP, green line). After the in vitro kinase assay with murine activated ERK2, 32P incorporation was determined by filter binding and scintillation counting, with values shown representing the average of two samples.

(B) Vmax and Km were determined from kinetic analyses from the in vitro kinase assays. The relative acceptor ratio (RAR) values were calculated from Vmax and Km and normalized to MBP. WT EOR-1 was robustly phosphorylated by ERK, as indicated by high RAR value. The RAR value of EOR-1 is significantly decreased by L81F.

(C) Graph of CPM versus protein concentration for GST-tagged EOR-1(WT) (yellow line), GST-tagged EOR-1(N) (purple line), GST-tagged EOR-1(C) (green line), GST-tagged LIN-1 truncated as positive control (orange line) or myelin basic protein (MBP, green line).

(D) RAR values were calculated as described in B. A truncated form of LIN-1 that contains amino acids 281-441 was used as the positive control (JACOBS et al. 1998). EOR-1N was robustly phosphorylated by ERK, as indicated by the high RAR value, whereas EOR-1C was moderately phosphorylated by ERK.

Figure 7. The EOR-1-EOR-2 interaction is direct and can occur in the absence of ERK phosphorylation

(A) Reducing Ras signaling with a sos-1 (ts) allele does not disrupt the interaction between EOR-1 and EOR-2. Similar levels of EOR-1 co-immunoprecipitated with EOR-2::GFP from eor-2 and eor-2; sos-1(ts) staged animals shifted to the restrictive temperature at the L2 stage.

EOR-1 did not co-immunoprecipitate from animals only expressing ttx-3p::GFP.
(B) EOR-1 and EOR-2 interact in vitro. In vitro translated 3xFLAG::EOR-2 bound to bacterially expressed GST::EOR-1 but not to GST alone (left). As a positive and negative control, in vitro translated Gal4DBD::EOR-1N but not Gal4DBD::EOR-1C bound to GST::EOR-1 (right).
FIGURE 1

A

EOR-1

| 9 Zn Fingers |
| D-Domain |

B

wild-type eor-1 (cs28) (null) eor-1 (cs44) (L81F)

C

Ce EOR-1

Cb EOR-1

Hs PLZF

Hs BCL-6

B1

β1

α1

B3

A3

β5

A5

A2

A1

A4

D

IB:

EOR-1

HSP90

E

wild-type eor-1 (cs28) (null) eor-1 (cs44) (L81F)
FIGURE 2

A

![Diagram showing cell division and genetics](image)

B

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Transgene</th>
<th>% WT P6.p division (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>-</td>
<td>100 (many)</td>
</tr>
<tr>
<td>lin-39*</td>
<td>-</td>
<td>100 (20)</td>
</tr>
<tr>
<td>eor-1*</td>
<td>-</td>
<td>100 (46)</td>
</tr>
<tr>
<td>lin-39; eor-1*</td>
<td>-</td>
<td>0.14</td>
</tr>
<tr>
<td>lin-39; eor-1</td>
<td>eor-1p::EOR-1::GFP</td>
<td>93 (27)**</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>0 (15)</td>
</tr>
<tr>
<td></td>
<td>eor-1p::EOR-1L81F::GFP</td>
<td>4 (24)</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>0 (26)</td>
</tr>
<tr>
<td></td>
<td>eor-1p::EOR-1S64A::GFP</td>
<td>71 (24)**</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>6 (17)</td>
</tr>
<tr>
<td></td>
<td>eor-1p::EOR-1S248A::GFP</td>
<td>86 (19)**</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>6 (17)</td>
</tr>
<tr>
<td></td>
<td>eor-1p::EOR-1S64A;S248A::GFP</td>
<td>65 (23)**</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>5 (22)</td>
</tr>
<tr>
<td></td>
<td>eor-1p::EOR-1S64A;S248A::GFP</td>
<td>65 (23)**</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>5 (20)</td>
</tr>
<tr>
<td></td>
<td>eor-1p::EOR-1S64A;S248A::GFP</td>
<td>55 (20)**</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>6 (18)</td>
</tr>
<tr>
<td></td>
<td>eor-1p::EOR-1S64A;S87A;S248A::GFP</td>
<td>35 (23)*</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>5 (19)</td>
</tr>
<tr>
<td>lin-31p::eor-1 line 1</td>
<td>-</td>
<td>91 (35)**</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>0 (31)</td>
</tr>
<tr>
<td>lin-31p::eor-1 line 2</td>
<td>-</td>
<td>86 (28)**</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>0 (29)</td>
</tr>
<tr>
<td>dpy-7p::eor-1 line 1</td>
<td>-</td>
<td>17 (30)</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>0 (22)</td>
</tr>
<tr>
<td>dpy-7p::eor-1 line 2</td>
<td>-</td>
<td>17 (30) *</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>0 (24)</td>
</tr>
<tr>
<td>dpy-7p::eor-1 line 3</td>
<td>-</td>
<td>4 (23)</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>0 (23)</td>
</tr>
<tr>
<td>lin-39; eor-2</td>
<td>eor-2p::EOR-2::GFP</td>
<td>88 (24)**</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>5 (21)</td>
</tr>
<tr>
<td></td>
<td>eor-2p::EOR-2C900S::GFP line 1</td>
<td>0 (22)</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>0 (22)</td>
</tr>
<tr>
<td></td>
<td>eor-2p::EOR-2C900S::GFP line 2</td>
<td>6 (18)</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>5 (20)</td>
</tr>
<tr>
<td></td>
<td>eor-2p::EOR-2C900S::GFP line 3</td>
<td>0 (20)</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>0 (22)</td>
</tr>
<tr>
<td>lin-31p::eor-2 line 1</td>
<td>-</td>
<td>88 (33)**</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>0 (32)</td>
</tr>
<tr>
<td>lin-31p::eor-2 line 2</td>
<td>-</td>
<td>92 (24)**</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>0 (32)</td>
</tr>
<tr>
<td>dpy-7p::eor-2 line 1</td>
<td>-</td>
<td>3 (31)</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>0 (30)</td>
</tr>
<tr>
<td>dpy-7p::eor-2 line 2</td>
<td>-</td>
<td>3 (30)</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>0 (30)</td>
</tr>
<tr>
<td>dpy-7p::eor-2 line 3</td>
<td>-</td>
<td>4 (23)</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>4 (23)</td>
</tr>
<tr>
<td>lin-39</td>
<td>eor-1p::EOR-1L81F::GFP</td>
<td>100 (24)</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>100 (24)</td>
</tr>
</tbody>
</table>
**FIGURE 4**

### A

Ce EOR-2
RAVSNLTVNAYLTHESLDDKDWTSRSRELETNKQCPGGLKKRIPVEPHKRNWIACAYTRL 832
Cb EOR-2
IgLALOKTVAYILSVENSHREHNKDNENKHSDVDEGEQGCEILKKRIPVEPHKRNWIACAYTRL 885
Dm CG17233
--RDNFEIYIQTMIGDIESFSFEEKDDEYFLGVSRSVNIMENCRR----KLLAITEKTRSTISS 1318
Hs NP_079001.2
---PERVYKIAFADEVQLIGTRRK-YSDKMTLYLDKREVQPRESLVSHKKEKYKER 405

Ce EOR-2
NISKSSFHCFEKT-----CQICK-RKKPRVRHHFDKTSCEKELVENSSDHIDMEDDEFYPVSBAISC 97
Cb EOR-2
NYSKAYSAYLCK-----CQICK-RKKPRRIHHFDKTYTLMVEKKPEFPTPEKMRSEIVADAILSC 947
Dm CG17233
JELEMPKCRFSS-----WQNN-LTKNCACCHGPGYVPRFLLEFENYNFMOTPPYPIVYERKLF 1383
Hs NP_079001.2
VENVNVSYHLKNEPCSQAGLHRCEYVSHELYNTRMLEDNMFH-----KQFYTVK-----G 465

Ce EOR-2
GRCSMAVDFLRHHHRKHFFHMLRIFED--KLEEIGTSDIDLTSDFKEVAKADHVLTVGLCYCKOLWQVR 966
Cb EOR-2
GRCASADEFHRHHRHMRFLHRLLKEYD--KLEEELGTTSIDLSVMEMEAKATTDKKWMLNELTDKHFHLWQVR 1016
Dm CG17233
RICAARADLFHKIAHDFKNFLFINSQ--RVIDEQQQQPPFGKTESILNDHMAEHNIDELFRNMBNCLREV-- 1451
Hs NP_079001.2
RICAERTRKHCFLKHFKLYGQCTIAMTVEDEQYKEVTERFFR-SKENGWIKEKYGPLEYLN----- 532

---

### B

**GAL4::EOR-1**

IB: GAL4::EOR-1

**FL::EOR-2**

IP GAL4

5% Input

---

### C

**GAL4::EOR-1**

IB: GAL4::EOR-1

**FL::EOR-2**

IP GAL4

5% Input

---

### D

**EOR-2 (aa1-973)**

**EOR-2 N (aa1-660)**

**EOR-2 C (aa661-973)**

**EOR-2(C900S)**

- Bind EOR-1?

---

### E

**Photograph**

- Arrowheads
FIGURE 5

A

IB: eor-2; EOR-2::GFP
eor-2; eor-1 (L81F); EOR-2::GFP
eor-2; eor-1 (null); EOR-2::GFP
eor-2; ttk-3p::GFP

IP GFP
2.5% Input

IB: EOR-1
IP GFP
2.5% Input

IB: HSP90
IP GFP
2.5% Input

B

IB: ttk-3p::GFP
eor-2; EOR-2::GFP

MEL-26
3.5% Input

IB: EOR-1
IP GFP
3.5% Input

D

IB: ttk-3p::GFP
eor-1 (null); EOR-1::GFP

HDA-1
3.5% Input

IB: EOR-1::GFP
IP GFP
3.5% Input

IB: HSP90
IP GFP
3.5% Input
Figure 6

(A) ERK2 mediated phosphorylation kinetics of EOR-1.

(B) Table showing Vmax (pmol PO4/min/pmol of ERK2) and Km (µM) for various proteins.

<table>
<thead>
<tr>
<th>PROTEIN</th>
<th>Km (µM)</th>
<th>Vmax (pmol PO4/min/pmol of ERK2)</th>
<th>RAR</th>
</tr>
</thead>
<tbody>
<tr>
<td>MBP</td>
<td>4.3 ± 0.5</td>
<td>57 ± 0.7</td>
<td>1</td>
</tr>
<tr>
<td>LIN-1 WT</td>
<td>0.18 ± 0.5</td>
<td>24 ± 0.8</td>
<td>10</td>
</tr>
<tr>
<td>EOR-1 WT</td>
<td>0.12 ± 0.09</td>
<td>19 ± 0.3</td>
<td>12</td>
</tr>
<tr>
<td>EOR-1(L81F)</td>
<td>0.42 ± 0.1</td>
<td>14 ± 0.1</td>
<td>3</td>
</tr>
</tbody>
</table>

(C) ERK2 mediated phosphorylation kinetics of EOR-1 deletion constructs.

(D) Table showing Vmax (pmol PO4/min/pmol of ERK2) and Km (µM) for various deletion constructs.

<table>
<thead>
<tr>
<th>PROTEIN</th>
<th>Km (µM)</th>
<th>Vmax (pmol PO4/min/pmol of ERK2)</th>
<th>RAR</th>
</tr>
</thead>
<tbody>
<tr>
<td>MBP</td>
<td>4.7 ± 0.5</td>
<td>61 ± 0.2</td>
<td>1</td>
</tr>
<tr>
<td>LIN-1 truncated</td>
<td>0.32 ± 0.1</td>
<td>18 ± 0.8</td>
<td>4</td>
</tr>
<tr>
<td>EOR-1WT</td>
<td>0.10 ± 0.6</td>
<td>21 ± 0.3</td>
<td>16</td>
</tr>
<tr>
<td>EOR-1N</td>
<td>0.18 ± 0.3</td>
<td>18 ± 0.2</td>
<td>8</td>
</tr>
<tr>
<td>EOR-1C</td>
<td>0.31 ± 0.1</td>
<td>16 ± 0.2</td>
<td>4</td>
</tr>
</tbody>
</table>
FIGURE 7

A

IB:

EOR-1

GAL4::EOR-1C

GAL4::EOR-1N

HSP90

IB:

FL::EOR-2

FL::EOR-2

B

GST:: empty GST:: EOR-1 3XFL:: EOR-2 EOR-2

IB:

GST pulldown

Input

GST:: GAL4:: EOR-1 EOR-1 EOR-1

IB:

GAL4::EOR-1C

GAL4::EOR-1N

GAL4::EOR-1C

GAL4::EOR-1N

Input

GAL4:: EOR-1N