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COMMUNICATION

# A transcription assay for EWS oncoproteins in *Xenopus* oocytes

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## ABSTRACT

**Aberrant chromosomal fusion of the Ewing's sarcoma oncogene (EWS) to several different cellular partners produces the Ewing's family of oncoproteins (EWS-fusion-proteins, EFPs) and associated tumors (EFTs). EFPs are potent transcriptional activators, dependent on the N-terminal region of EWS (the EWS-activation-domain, EAD) and this function is thought to be central to EFT oncogenesis and maintenance. Thus EFPs are promising therapeutic targets, but detailed molecular studies will be pivotal for exploring this potential. Such studies have so far largely been restricted to intact mammalian cells while recent evidence has indicated that a mammalian cell-free transcription system may not support bona fide EAD function. Therefore, the lack of manipulatable assays for the EAD presents a significant barrier to progress. Using *Xenopus laevis* oocytes we describe a plasmid-based micro-injection assay that supports efficient, bona fide EAD transcriptional activity and hence provides a new vehicle for molecular dissection of the EAD.**

**KEYWORDS** EWS/ATF1, Ewing's sarcoma, microinjection, *Xenopus* oocytes, transcription, EWS-activation domain

## INTRODUCTION

Chromosomal translocations involving the Ewing's sarcoma oncogene (EWS) or the related genes TAF15 and TLS/FUS (the TET family, Law et al., 2006) give rise to the Ewing's family of oncoproteins (EWS-fusion-proteins, EFPs) and their associated tumors (EFTs; Kim and Pelletier, 1999; Arvand and Denny, 2001; Janknecht, 2005). EFPs include EWS/FLI1 (EFT: Ewings sarcoma), EWS/WT1 (desmoplastic small round cell tumor), EWS/ATF1 (clear cell sarcoma), EWS/TEC (chondrosarcoma), EWS/ZSG (small round cell tumor), TLS/ERG (myeloid leukemia) and TLS/CHOP (liposarcoma). EFPs are gene-specific transcriptional activators dependent on the N-terminal region of EWS (the EWS-activation-domain, EAD) and a C-terminal DNA binding domain provided by the particular fusion partner (Kim and Pelletier, 1999). Transcriptional de-regulation by EFPs is most likely central to EFT oncogenesis but other effects of EFPs, including transcriptional repression or perturbation of pre-mRNA splicing are also likely to be important (Arvand and Denny, 2001).

EFPs may be promising therapeutic targets (Kovar et al., 1999) because they are absolutely tumor specific and their function is quite distinct from the parental TET proteins (Arvand and Denny, 2001). In addition at least in some cases (Prieur et al., 2004; Davis et al., 2006) EFPs appear to play a role in tumor cell survival, indicating the therapeutic potential of EFP inhibitors. Detailed biochemical analysis of EFPs will be essential for exploration of the above possibility and the EAD is of particular interest because it is common to the entire EFT family.

Transient introduction of exogenous EFPs into mammalian tissue culture cells has uncovered some essential structure/function relations for the EAD (Feng and Lee, 2001; Ng et al., 2007). Notably, systematic mutational analysis of intact EAD showed that multiple dispersed Tyr residues are crucial for EAD function in mammalian cells (Ng et al., 2007). Of relevance to the current study, the availability of informative EAD mutants (Fig. 1C) provides an essential tool for evaluating novel assays for the EAD. Despite the above progress several other factors hindered biochemical characterization of the EAD. First, the EAD is complex (spanning ~250 residues) and harbors dispersed functional elements (Pan et al., 1998). Second, the highly biased composition of the EAD (resulting from the presence of a degenerate hexapeptide repeat [DHR] with consensus SYGQQS, Fig. 1B) confers the

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properties of an intrinsically disordered protein (IDP) region (Ng et al., 2007) and thus precludes classical structural analysis. Third, the EAD most likely interacts with a complex array of proteins (Rual et al., 2005) and identification of critical EAD partners has not been achieved.

One further barrier to biochemical studies of the EAD is that available functional assays in mammalian cells and yeast (Zhou and Lee, 2001) are not easy to manipulate. Crucially it has also been shown that mammalian cell extracts do not support bona fide EAD function (Ng et al., 2009). Development of more tractable experimental systems to study the EAD is therefore of significance. *Xenopus leavis* oocytes offer some advantages as a heterologous host system for mammalian transcription factors. Exogenous mammalian promoters can be activated by the endogenous oocyte transcription machinery (Gurdon and Wickens, 1983; Gurdon and Wakefied, 1986). In addition, PolII promoters are active at low template levels in oocytes (Gurdon and Wakefied, 1986) and are efficiently assembled into chromatin thus favoring support of regulated events (Gurdon and Wickens, 1983). Finally, the large size of oocytes (relative to mammalian cells) readily allows for microinjection of macromolecules (RNA, proteins/antibodies and peptides) and thus greatly facilitates testing of potential inhibitors. Here we describe the use of *Xenopus* oocytes and a plasmid-based micro-injection assay that supports efficient, bona fide EAD transcriptional activity and thus provides a new vehicle for molecular dissection of the EAD.

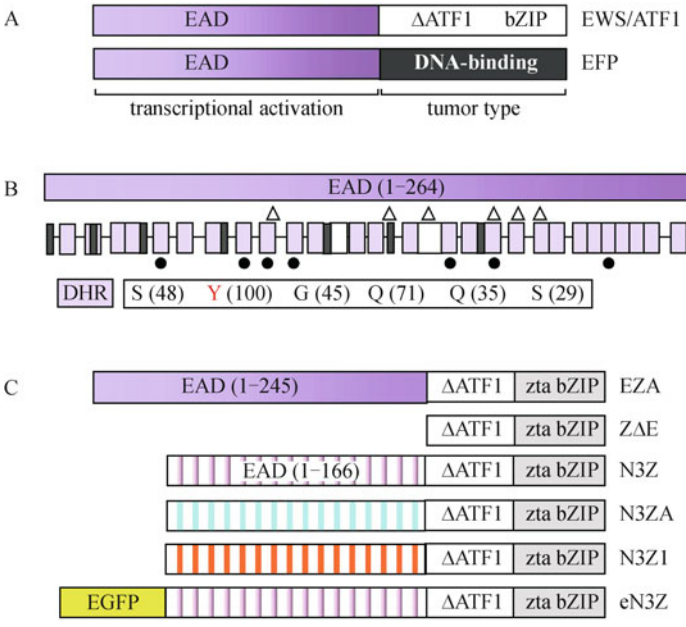


**Figure 1. Structures of EWS/ATF1 (EFPs), EAD, and experimental activators and reporters.** (A) Structure of EWS/ATF1 (EFPs). The Ewing's family of oncoproteins (EFPs) contains the N-terminal region of the Ewing's sarcoma oncogene (EWS) fused to various transcription factor partners (Kim and Pelletier, 1999). All EFPs contain at least EWS residues 1–264 (the EWS-activation-domain, EAD) and a sequence-specific DNA binding domain from distinct partners. EWS/ATF1 is a typical EFP and contains EWS residues 1–325 fused to the C-terminal region of ATF1 (ΔATF1, residues 66–271), including the DNA binding (bZIP) domain (Zucman et al., 1993). EWS/ATF1 is a potent constitutive activator of ATF-dependent promoters (Ng et al., 2007) dependent on the EAD and the bZIP domain of ATF1. (B) Primary structure of the EAD. The EAD contains multiple degenerate hexapeptide repeats (DHRs, purple boxes) with consensus sequence SYGQQS. DHR degeneracy is indicated by the % occurrence of conserved residues including the absolutely conserved Tyr residue (red). Seven additional Tyr residues (dark gray boxes) are also present. Spaces between DHRs are generally only a few residues except in two cases (white boxes) of 12 and 25 residues, respectively. Several SH2 binding sites (YxxP, black circle) and SH3 binding sites (PxxP, open triangle) are indicated. (C) Structure of experimental activators and reporters. EZA contains the intact EAD (EWS residues 1–245), ΔATF1 and the zta bZIP domain (Ribeiro et al., 1994), ZΔE is equivalent to EZA but lacks the EAD. N3Z contains residues 1–166 of the EAD but is otherwise equivalent to EZA. Several Tyr residues within DHRs (purple boxes in N3Z) that are critical for transcriptional activation in mammalian cells (Ng et al., 2007) are shown. N3ZA and N3ZI are essentially the same as N3Z except that the Tyr residues highlighted (see materials and methods for precise coordinates) are changed to Ala (N3ZA, blue) or Isoleucine (N3ZI, orange). eN3Z corresponds to N3Z with EGFP fused to the N terminus and likewise for eN3ZA and eN3ZI (not shown). Expression vectors pSVEZA (Li and Lee, 2000), pZΔE (Feng and Lee, 2001), pN3Z, pN3ZA and pN3ZI (Ng et al., 2007) were described elsewhere. All expression vectors were derived from pSG424 (Sadowski and Ptashne, 1989) containing the SV40 early promoter and polyadenylation signals for expression in *Xenopus* oocytes. All proteins contained the KT3 epitope PPPEPET (MacArthur and Walter, 1984) at the C-terminus.


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## RESULTS AND DISCUSSION

To test EAD function in oocytes, we exploited EWS/ATF1 (Brown et al., 1995) or close derivatives (Ng et al., 2007) that serve as useful EFP models. EWS/ATF1 is a potent activator in mammalian cells (Brown et al., 1995) and, crucially, we have recently characterized informative EAD mutants (Fig. 1C) in this context (Ng et al., 2007). Because oocytes contain endogenous CREB/ATF-related activators that could impact EWS/ATF1 activity (either via binding to ATF sites in the reporter or via heterodimerization) we employed a subtle domain swap (Ribeiro et al., 1994) involving substitution of the ATF1 bZIP domain with that of the EBV zta protein (Fig. 1C). Promoters containing multiple zta binding sites fused to CAT (Z7E4TCAT, referred to as Z7CAT) or Luciferase (Z7Luc) reporters give essentially background activity in oocytes (Fig. 2) thereby allowing response to exogenous activator.

EZA (Fig. 2) contains essentially the intact EAD (EWS residues 1–245) fused to the part of ATF1 present in EWS/ATF1 (ΔATF1) and the zta-bZIP domain. Initially we asked whether EZA could activate the Z7CAT reporter in oocytes. An expression vector for EZA driven by the SV40 early promoter that is active in oocytes (Jones et al., 1983) and Z7CAT reporter plasmid were co-injected into stageVI oocyte germinal vesicles (GV) and CAT assays (using pooled extracts, each from three oocytes) performed 40 h post-injection. Compared with Z7CAT reporter alone, EZA significantly activated transcription (48-fold) suggesting that the EAD is functional in the oocytes (Fig. 2).

The CAT reporter assay is relatively insensitive/time consuming and so we employed Luciferase to improve the assay and test EAD-dependence. EZA stimulates transcription of the pZ7Luc reporter ~180-fold (Fig. 2 and Table 1 Exp#1) and deletion of the EAD (ZΔE) reduced activation to ~8-fold (Fig. 2 and Table 1 Exp#1). Western blotting of extracts (pooled from seven oocytes) using KT3 antibody (Fig. 2) indicates that (consistent with results in mammalian cells) EZA is expressed at lower levels than ZΔE and thus the effect of the EAD in oocytes may be underestimated. To assess the absolute magnitude of trans-activation by EZA we injected oocytes with pGL3 which contains the SV40 early promoter expressing Luciferase (Fig. 2) and found that EZA activity was generally about 2–4 fold less than that of pGL3 (see also Fig. 4 for eN3Z). Together the above results show that the EAD can efficiently activate transcription in *Xenopus* oocytes.

Due to low expression of EZA or the relative sensitivity of the KT3 antibody, it was not possible to scrutinize individual oocytes or determine the correlation between EZA levels and activity. We therefore sought to sensitize the assay and establish data for individual oocytes. Previously we have shown in mammalian cells that proteins containing the intact EAD (such as EZA) are expressed at lower levels than those with deletions of the C-terminal region of EAD (Pan et al., 1998). Thus a protein containing EAD residues 1–166 (N3Z, Fig. 1C) is expressed at higher levels than EZA and remains a potent activator in mammalian Jeg3 cells (Fig. 3A). To confirm that introduction of the zta-bZIP domain in N3Z does not affect EAD activity we employed a mutant protein N3ZA (Fig. 1C) that harbors several Tyr to Ala substitutions (Fig. 1C) known to inactivate the EAD (Ng et al., 2007). N3ZA is transcriptionally inactive in Jeg3 cells as expected (Fig. 3A) but is expressed at high levels and retains full DNA binding activity (Fig. 3A). Characterization of several EAD mutants (Ng et al., 2007) has established that the effect of mutations present in N3ZA (or N3ZI, Fig. 1C) reflects the crucial role of Tyr residues in EAD activity and does not reflect a gross protein malfunction engendered by the significant mutational burden. Specifically a similar degree of Ala substitutions in non-tyrosine EAD residues (Gln to Ala or Ser/Thr to Ala) does not

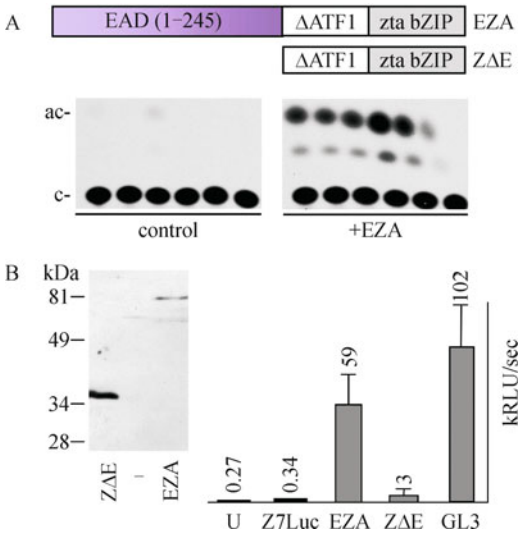


**Figure 2. EAD-mediated trans-activation in *Xenopus* oocytes.** (A) The germinal vesicle of unfertilised stage VI *Xenopus* oocytes was injected with 2 ng of a Cat reporter plasmid (pZ7E4TCAT) containing seven zta binding sites either alone (control) or in the presence of 2 ng of vector (pSVEZA) expressing EZA (+ EZA) containing the intact EAD. Cat assays were performed 40 h post-injection and an autoradiogram of the CAT assay is shown (c, chloramphenicol; ac, acetylated chloramphenicol). Each sample in the Cat assay shows the activity from three injected oocytes pooled together. (B) Oocytes were injected with 2 ng of a Luciferase reporter (pZ7Luc) containing seven zta binding sites and expression plasmids (2 ng) for EZA and ZΔE or pGL3 (an SV40 promoter-Luciferase reporter). Luciferase reporter activity (kRLU/sec ± SEM) was determined for individual oocytes and the mean values are shown (see Table 1 Exp#1) for uninjected oocytes (U), pZ7Luc alone, EZA, ZΔE and pGL3. Western blot using KT3 antibody (left hand side) shows expression levels for EZA and ZΔE. Extract equivalent to 7 oocytes (derived from a total of 34 oocytes) was loaded on the gel.

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**Table 1** Data summary

| | activator | No. oocytes | Luc. activity | fold activation |
|---|---|---|---|---|
| Exp. #1 | uninjected | 4 | 0.27 ± 0.01 | − |
| | pZ7Luc alone | 17 | 0.34 ± 0.02 | − |
| | GL3 | 11 | 102 ± 29 | − |
| | EZA | 15 | 59 ± 18 | 174 |
| | ZΔE | 26 | 2.8 ± 0.3 | 8 |
| Exp. #2 | N3Z | 20 | 86 ± 19 | 254 |
| | N3ZA | 20 | background | 1 |
| Exp. #3 | N3Z | 6 | 56 ± 12 | 165 |
| | eN3Z | 5 | 125 ± 41 | 368 |
| Exp. #4 | eN3Z | 10 | 78 ± 17 | 229 |
| | GL3 | 10 | 214 ± 58 | − |
| Exp. #5 | eN3Z | 10 | 41 ± 15 | 121 |
| | eN3ZA | 10 | background | 1 |
| | eN3ZI | 10 | background | 1 |

The table shows the activator protein in each experiment (activator), number of oocytes analyzed (No. oocytes), transcriptional activity (Luc. activity, kRLU/sec ± SEM) and fold activation compared with the pZ7Luc reporter alone (fold activation).

inactivate the EAD (Ng et al., 2007). Thus, the Tyr to Ala or Ile mutations (Fig. 1C) enable verification of authentic EAD activity in other systems. We tested N3Z in *Xenopus* oocytes and detected high levels of trans-activation in individual oocytes (Fig. 3B and Table 1 Exp#2). The large variation for N3Z activity in different oocytes is consistently observed (see also Fig. 4) and is discussed later. N3ZA gives only background levels of activity in oocytes (Fig. 3B and Table 1 Exp#2) and thus the above results indicate that the oocyte assay reflects authentic EAD function.

Although N3Z is expressed at higher levels than EZA, we were still unable to readily detect N3Z expression in individual oocytes using KT3 antibody (data not shown). To overcome the above obstacle we produced enhanced green fluorescent protein (EGFP) derivatives (eN3Z, eN3ZA and eN3Z1, Fig. 1C) and exploited a very sensitive EGFP antibody (JL8 antibody, Clontech). eN3Z strongly activates transcription in mammalian Jeg3 cells as control (Fig. 3C) and the corresponding EAD mutant proteins eN3ZA and eN3ZI (Fig. 1C) are defective, as expected (0.1% and 0.4% of eN3Z activity, respectively). Western blotting using anti-EGFP JL8 antibody confirms expression eN3ZA and eN3ZI proteins (Fig. 3C) and N3ZA expression is also elevated relative to eN3Z (but less so than in the case of N3ZA versus N3Z, Fig. 3A). The above results show that eN3Z-derivatives behave appropriately in mammalian cells and can serve as reliable tools for studying the EAD in oocytes (or in other systems). eN3Z strongly activated the Z7Luc reporter in oocytes (Fig. 3C and Table 1 Exps#3–5). In one side-by-side test eN3Z was ~2-fold more active than N3Z (Fig. 3C and Table 1 Exp#3). As expected from their lack of activity in mammalian cells, eN3ZA and eN3ZI had no detectable activity in oocytes (Fig. 3C).

Significantly, eN3Z protein (and eN3ZA/eN3ZI) can readily be detected in single oocytes by Western blotting using the anti-EGFP JL8 antibody (Fig. 4) and this allowed correlation of activator levels and transcriptional activity in individual oocytes. Ten oocytes were injected with pGL3 (as a robust indicator of transcriptional competence in the oocyte population) or co-injected with the Z7Luc reporter and eN3Z, eN3ZI or eN3ZA. eN3ZI and eN3ZA are both expressed in oocytes (Fig. 4) and thus the lack of activity for eN3ZA and eN3ZI in oocytes is not explained by poor expression. Trans-activation by eN3Z is detectable in ~80% of injected oocytes but varies over a wide range for individual oocytes (Fig. 4). The above result is typical and is reflected by a similar and relatively large SEM (22%–36% of the mean values) in three different experiments (Table 1, Exps#3–5). The small number of inactive oocytes that do not express eN3Z is probably due to poor injection (Gurdon and Wakefield, 1986; Guille, 1999). For active oocytes there is not a particularly strong correlation between activator (eN3Z) levels and transcription activity. However, variability in results for the SV40 promoter (pGL3) is similar to that for eN3Z with SEMs equal to 27% and 28% of the mean value in two experiments (Table 1, Exps#1 and 4). This variation is within expectation (Gurdon and Wickens, 1983) since similar variation also occurs for simple tests (GL3 in our experiments) requiring activation of injected PolII promoters by endogenous oocyte factors (Gurdon and Wickens, 1983). Thus, variable EAD activity reflects general experimental/oocyte variation rather than the existence of specific problems related to EAD-mediated trans-activation.

## CONCLUSION

The DNA-based oocyte micro-injection assay described

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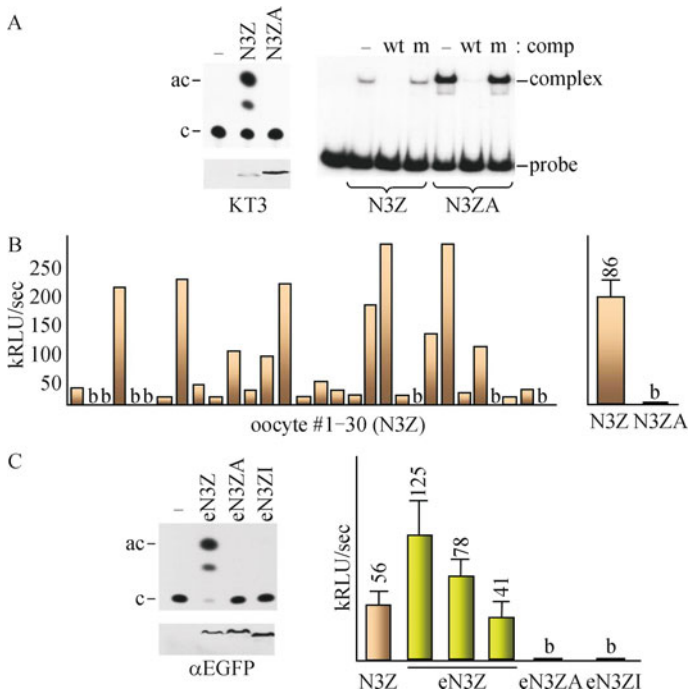


**Figure 3. Analysis of N3Z and EGFP-N3Z proteins.** (A) Transcriptional activity of N3Z and N3ZA in mammalian cells was detected by Cat assay using the Z7Cat reporter. Western blot analysis using KT3 antibody shows activator expression levels. DNA binding activity was detected by gel mobility shift assay (right hand side). Extracts from transfected cells were incubated with 1ng of labeled probe containing a single zta binding site. DNA-protein complexes were resolved on non-denaturing polyacrylamide gels and detected by autoradiography. Positions of unbound DNA probe and DNA-protein complexes are indicated to the right. The presence of excess competitor oligonucleotide (100 ng) containing a functional (wt) or mutated (mt) zta binding site is indicated. (B) Transcriptional activity (kRLU/sec ± SEM) of N3Z and derivatives in oocytes was determined as described in Fig. 2 using the Z7Luc reporter. The figure shows the individual data for each injected oocyte (left hand plot) and the mean values with SEM (right hand side, see Table 1 Exp#2). (C) Transcriptional activity of EGFP-N3Z derivative (eN3Z) and mutants eN3ZA and eN3ZI was determined in mammalian cells by Cat assay using the Z7Cat reporter (left hand side). A Western blot using αEGFP antibody JL8 (Clontech) shows activator expression levels. The right hand graph shows the activity of eN3Z in oocytes using the Z7Luc reporter (mean values and SEM are shown, see Table 1 Exps#3–5). eN3ZA and eN3ZI both gave only background activity (b).

herein supports efficient, bona fide EAD transcriptional function. Even the lower range of N3Z activity is ~100-fold higher than the Z7Luc reporter background thus providing a broad window for experimentation. Although there is systemic variability, robust data can typically be obtained by averaging the N3Z activity from as few as ten oocytes. This is comparable with other transcription assays employing injected oocytes (Gurdon and Wickens, 1983).

Considering the advantage of single cell experiments using *Xenopus* oocytes, the likely conservation of cellular pathways relevant to the EAD (see below) and the indication that simple mammalian cell-free transcription systems do not support EAD function (Ng et al., 2009), *Xenopus* oocytes may provide advantages for biochemical dissection of the EAD. Most notably compared with mammalian cells, oocytes can be readily microinjected (with RNA, proteins/antibodies and peptides) using relatively simple equipment and this will greatly facilitate inhibitor studies. The EAD is a potential target for drug design and the ability to test macromolecular inhibitors under well defined conditions will enable effective evaluation of early lead compounds. With regard to development of a soluble transcription system for the EAD, it remains to be seen whether oocyte extracts will faithfully support EAD activity or whether they will exhibit the same deficiency as mammalian cell extracts (Ng et al., 2009).

The EAD is not broadly conserved in the animal kingdom but is highly conserved between frogs, zebra fish (Azuma et al., 2007) and mammals, including multiple Tyr phosphorylation sites and SH2/SH3 binding motifs (Fig. 1B). Frog oocytes are thus likely to harbor crucial endogenous factors that have co-evolved with the EAD and this should be advantageous over alternative (non-mammalian) systems for EAD analysis, such as yeast (Zhou and Lee, 2001). For example Tyr phosphorylation can modulate EAD activity under particular circumstances (Kim et al., 1999, 2000) and other phosphorylation events (Olsen and Hinrichs, 2001) and O-GlcNAcylation (Bachmaier et al., 2009) have been shown to influence EFP/EAD activity.

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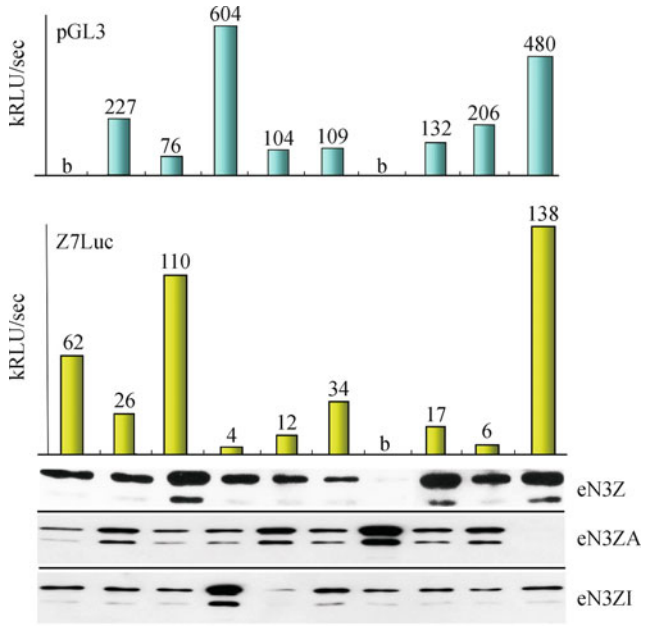


**Figure 4. Analysis of activator levels and EAD activity in individual oocytes.** For pGL3 control reporter (blue bars, average activity 214 ± 58 kRLU/sec) and activation of Z7Luc by eN3Z (green bars, average 78 ± 17 kRLU/sec) the activity detected in ten individual oocytes is shown. The corresponding level of eN3Z expression for each oocyte (detected by Western blot using αEFGP JL8 antibody) is shown below the activity graph. Expression levels for eN3ZA and eN3ZI in individual oocytes (detected using αEFGP) is shown for the same experiment as eN3Z. eN3ZA and eN3ZI yield only background signals for trans-activation (Fig. 3C).

## MATERIALS AND METHODS

### Plasmids

All expression vectors were derived from pSG424 (Sadowski and Ptashne, 1989) containing the SV40 early promoter and polyadenylation signals that allow expression in both mammalian cells and *Xenopus* oocytes. Expression vectors pSVEZA (Li and Lee, 2000) and pZΔE (Feng and Lee, 2001) are previously described. pN3Z was derived from pΔ167C (Pan et al., 1998) by replacing the ATF1 bZIP domain with the zta bZIP domain using an Nde1 site engineered for bZIP domain swaps (Ribeiro et al., 1994). pN3ZA and pN3ZI were obtained by inserting *Hin*dIII/*Bgl*II ended synthetic DNA fragments obtained by total gene synthesis (TOP Gene Technologies, Montreal) directly into pZΔE digested with *Hin*dIII/*Bgl*II. pZ7E4CAT (Carey et al., 1992) and pGL3-control vector (Promega) are described elsewhere. pZ7Luc was obtained by inserting a *Hin*dIII/*Kpn*I fragment from pZ7E4TCAT into pVIPRSVluc vector (Masson et al., 1992). peN3Z was prepared by joining a AgeI/*Sal*I fragment from pEGFPC-3 (Clontech) and a *Hin*dIII/*Sal*I fragment from pN3Z, using an oligonucleotide with *Hin*dIII/AgeI overhangs. peN3ZA and peN3ZI were obtained by replacing the wild type EAD sequence between *Hin*dIII/*Bgl*II in peN3Z with Hind3/Bgl2 ended synthetic DNA fragments harboring the desired mutations. All proteins contained the KT3 monoclonal epitope PPPEPET MacArthur and Walter, 1984) at the C-terminal adjacent to the zta bZIP domain.

### EAD mutants

The EAD mutations exploited in this study (Fig. 1C) have been functionally characterized in mammalian cells in the context of the natural EFP, EWS/ATF1 (Ng et al., 2007). The above proteins have multiple Tyr to Ala (N3ZA and eN3ZA) or Tyr to Ile (eN3ZI) changes dispersed throughout the EAD (corresponding to Tyr residues 9, 18, 29, 36, 44, 52, 61, 70, 77, 86, 93, 112, 118, 127, 158 and 165 in the normal EWS protein). Characterization of several other EAD mutants (Ng et al., 2007) have established that the effect of the Tyr to Ala/Ile mutations reflects the critical role of multiple Tyr residues in EAD activity, as opposed to a gross protein malfunction engendered by the mutational burden. Specifically, a similar degree of Ala substitutions in non-tyrosine EAD residues (Gln to Ala or Ser/Thr to Ala) do not inactivate the EAD (Ng et al., 2007). Thus, the Tyr to Ala/Ile mutants are valuable tools for verifying authentic EAD activity in other systems.

### Oocyte preparation

Anesthesia of frogs using 0.1% ethyl *m*-aminobenzoate, surgery and oocyte maintenance prior to injection were broadly according to established procedure (Goldin, 1992). Lobes were washed extensively in calcium-free OR2 medium (OR2MC, contains 82.5 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$ and 5 mM Hepes, adjusted to pH 7.5 using NaOH), placed in a 50 mL tube containing 20 mL of collagenase (1 mg/mL; Sigma C5138; 388 U/mg [collagen digestion activity] or 0.9 U/mg FALGPA hydrolysis activity) in OR2MC and incubated at room temperature with very gentle agitation on a rocking platform for 20 min. The collagenase solution was then removed, oocytes washed in calcium-free OR2 medium and then incubated in fresh collagenase for a further 40 min. Separated oocytes were further washed in OR2MC, stage VI oocytes were selected and kept at 18°C and at low density (about 20 oocytes per 10 cm petri dish) in Modified Barths solution (MBS).

### Microinjection

Visually healthy oocytes were injected the day after surgery. Preparation of needles and microinjection were performed by established procedures (Guille, 1999). Needles with external diameter of 12–15 μm were used for the microinjection and were calibrated by filling with trypan blue and injecting into light mineral oil using a Medical Systems Corporation PLI-100 micro-injector. For experiments ~10 nL of DNA sample in water (containing 2 ng of activator plasmid and 2 ng of reporter plasmid) was injected into the oocyte germinal vesicle (GV) and this was commonly achieved using an injection pressure of 10–15 psi for 60–l00 ms. Use of 4 ng of injected plasmid is just sub-saturating (Gurdon and Wickens, 1983) and should be optimal for transcriptional studies. Typically 10–20 oocytes were injected for each test and following injection, individual oocytes were kept in isolation at 18°C in MBS. Success rate for GV injection and subsequent oocyte viability was > 80% as expected (Gurdon and Wakefield, 1986; Guille, 1999) and reporter activity was assayed 40 h post-injection. Mean values ± the standard error (SEM) were calculated and presented in the results summary Table 1.

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**Oocyte reporter assays**

Injected oocytes were lysed by vigorous resuspension in 100 µL of MBS using a micropipette yellow tip. Cell extract supernatant was obtained by centrifugation at 12,000 rpm for 5 min in an Eppendorf microfuge. Luciferase assays were performing by mixing 50 µL of extract with 50 µL of Steady-Glo Luciferase substrate (Promega).

**Mammalian reporter assays**

Freshly passaged cells were transfected by calcium phosphate co-precipitation method as previously described (Brown et al., 1995) with 5 µg of plasmid expressing pN3Z/peN3Z (or EAD mutants), 5 µg of pZ7E4TCAT as reporter and 15 µg of pGem3 as carrier. CAT assays were performed at 40 h post-transfection as previously described (Brown et al., 1995).

**DNA binding assays**

Gel mobility shift assays using extracts from mammalian cells were performed as previously described (Ribeiro et al., 1994; Krajewski and Lee, 1994). The labeled zta DNA probe contains a single zta binding site with the TTGCTAA core motif and competitor oligonucleotides have either a wt zta binding site or a mutant site with the core motif changed to GACACAC. 15 µL binding reactions contained ~1.5 ng of labeled DNA probe and 1 µg of poly(dIdC) and were incubated for 20 min at 30°C. Half of the reaction mixture was resolved on 5% polyacrylamide gels run in 50% TBE. Gels were fixed in 50% methanol and exposed at minus 80°C for ~ 2 h against Kodak Biomax XAR film.

**Western blotting**

Western blotting was performed in PBS containing 3% dried milk. Tagged proteins were detected either using primary antibody KT3 (MacArthur and Walter, 1984) and alkaline phosphatase conjugated anti-mouse secondary antibody (DAKO D0486) or EGFP antibody (mouse monoclonal JL8, Clontech) and anti-mouse HRP conjugated secondary antibody (Amersham NA931) and detection using an ECL kit (Amersham NA931).

## ACKNOWLEDGEMENTS

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## ABBREVIATIONS

EWS, Ewing's sarcoma oncogene; EFP, EWS-fusion-protein; EAD, EWS-activation-domain; EFT, EWS-family-tumor; IDP, intrinsically disordered protein; DHR, degenerate hexapeptide repeat; MBS, Modified Barths solution; GV, germinal vesicle; CAT, chloramphenicol acetyl transferase; SEM, standard error of the mean

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