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Eos: a novel member of the Ikaros gene family expressed predominantly in the developing nervous system

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Abstract We identified a novel member of the Ikaros gene family, which has critical roles in the development of lymphoid lineages. This gene, which we named Eos, was expressed predominantly in the developing central and peripheral nervous system. Eos protein could interact with itself and Ikaros protein through its C-terminal portion in the yeast two hybrid assay. These findings suggested that Eos may have important roles in neural development similarly to the Ikaros family in the development of hemolymphoid tissue.

Key words: Ikaros family; Molecular cloning; Zn finger; Transcription factor; Nervous system; Development

1. Introduction

The Ikaros gene, which encodes a hemopoietic-specific zinc finger protein, is one of the central regulators of lymphocyte differentiation [1]. Loss of function experiments suggested that it is required at the earliest stage of B-cell and T-cell specification during fetal development and that lymphoid lineages rely on Ikaros at distinct phases of their development in adults [2,3]. In addition, Ikaros null thymocytes proliferate aggressively in response to T-cell receptor signalling, suggesting that Ikaros is a tumor suppressor gene for the T-cell lineage [4]. Ikaros isoforms unable to bind DNA exhibited dominant negative activity in lymphocyte development, suggesting that Ikaros works in concert with other factors [4,5]. Indeed, two structurally related genes, Aiolos and Helios, have been identified and shown to have lymphoid-restricted patterns of expression [6–8]. These factors were shown to form a complex regulatory protein network that controls cell fate decisions and regulates homeostasis in the hemolymphoid system (reviewed in [9,10]). The C-terminal Zn finger domain is indispensable for these protein-protein interactions [11].

We have been screening genes that are specifically up-regulated in the long term cultured mouse astrocytes (in vitro gliosis model) by differential display in order to identify gliosis-related genes. During the course of this screening, we identified a cDNA fragment with a significant sequence homology to mouse Ikaros family members. In the present study, we characterized the structure and the expression pattern of this novel member of the Ikaros family, which we named Eos (Greek word for rising sun).

2. Materials and methods

Astrocyte-enriched cultures were established from newborn mouse brains (ICR strain) as described previously [12]. The periods for short and long term cultures were 2 and 12 weeks, respectively. Total RNA was isolated by the acid guanidinium isothiocyanate/phenol-chloroform method from both long and short term cultured astrocytes. Differential display was carried out essentially as described previously [13]. We used about 100 different arbitrary primers (17-mers) for screening. After separation by 5% polyacrylamide gel electrophoresis, cDNA bands of interest were cut out from the gels, re-amplified with the same primers and then cloned into the pGEM-T Easy vector (Promega). DNA sequencing of the cloned fragments was performed using a Taq dye terminator cycle sequencing kit (Perkin-Elmer).

In order to obtain a full length cDNA, the cDNA fragment identified by differential display was random prime-labelled and used to screen 1×10^6 plaques of a mouse whole brain λgt10 cDNA library (Clontech) and a neonatal mouse brain λZAPIIcDNA library (Stratagene). Standard phage screening techniques were employed. For Northern analysis, total RNAs (20 μg) from the brain and liver of the developing (embryonic day 15 (E15), E18, postnatal day 0 (P0), P7 and adult) mice and from the spleen, kidney, lung and thymus of P0, P7 and adult mice were denatured with glyoxal and fractionated by electrophoresis through 1.0% agarose/formamide gels and transferred onto Zeta-Probe membranes (Bio-Rad). All other procedures were as described previously [13]. In situ hybridization using digoxigenin-labelled cRNA probes was performed as described previously [14]. Digoxigenin-labelled probes (antisense and sense probes) were made by in vitro transcription using cDNAs subcloned into the pGEM-T Easy vector as templates in the presence of digoxigenin-labelled dUTP.

We performed the following yeast two hybrid assay using a Matchmaker kit (Clontech Laboratories) according to the manufacturer's protocol. Full length mouse Eos as well as cDNA fragments encoding the N-terminal half (Eos-N) and C-terminal half of Eos (Eos-C) (see Fig. 3) were cloned in frame to the GAL4 DNA binding domain in the bait pGBT9 plasmid using standard molecular techniques. Full length Eos and human Ikaros genes were also cloned in frame to the GAL4 transcription activation domain of the prey vector pGAD424. Bait and prey vectors were transformed into the SFY526 yeast strain and the transformed yeast was grown on plates without tryptophan and leucine to select double transformants. The selected colonies were then subjected to a β-galactosidase assay. The ability of Eos and Eos fragments expressed in the bait vectors to activate lacZ gene expression was determined by the extent of color reaction on filters soaked with X-gal solution.

3. Results

We isolated a cDNA fragment (about 1.0 kb in size) during differential display screening of genes that were up-regulated in long term cultured astrocytes. Its partial sequence was de-

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termined and a database search revealed a significant homology to Ikaros, a Zn finger transcription factor (data not shown). By a RNase protection assay, the gene expression was confirmed to be up-regulated in long term cultured astrocytes (data not shown). To obtain the full length cDNA of this gene, we screened a cDNA library with the cDNA fragment identified by differential display and isolated two overlapping cDNAs. Their nucleotide sequences were determined

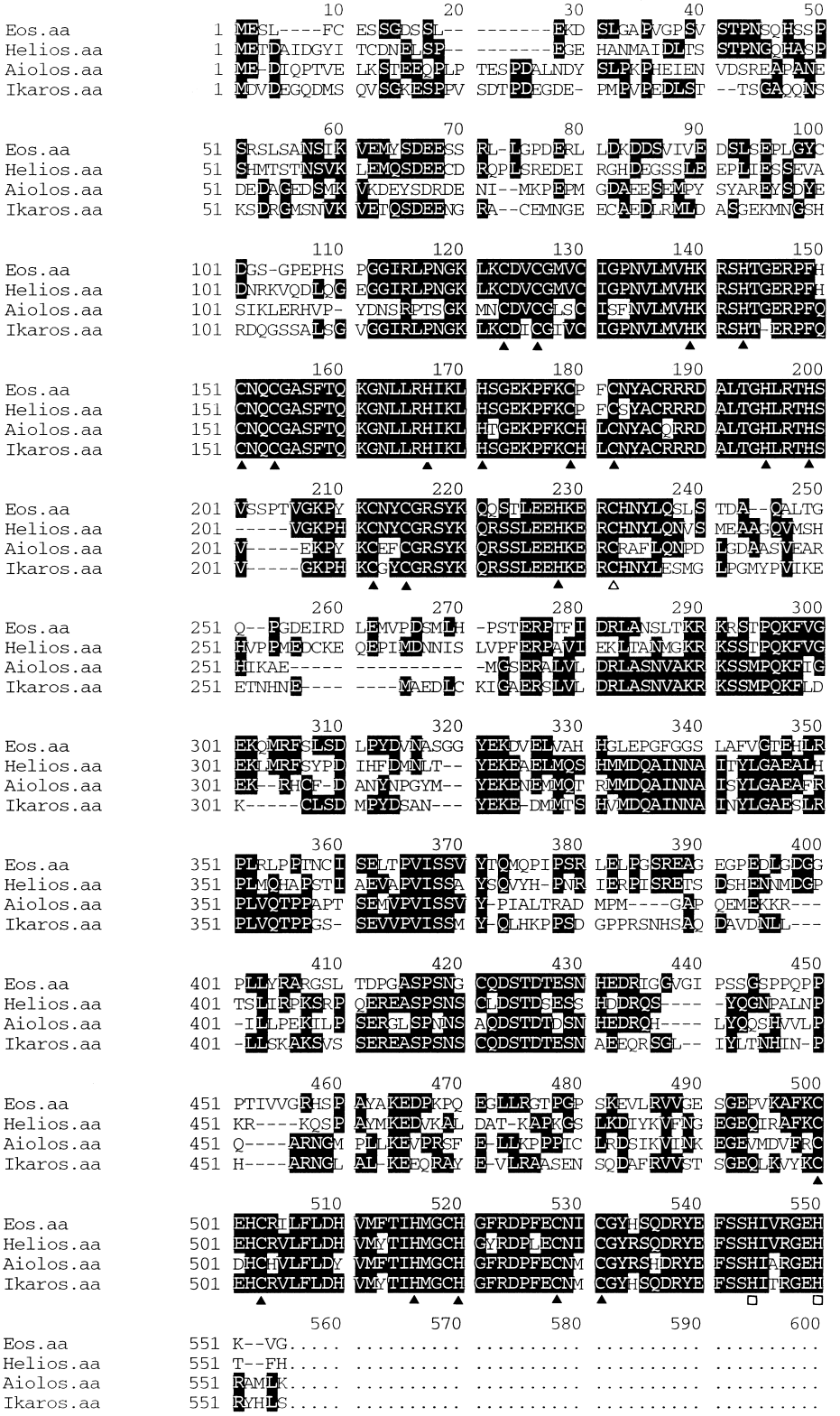
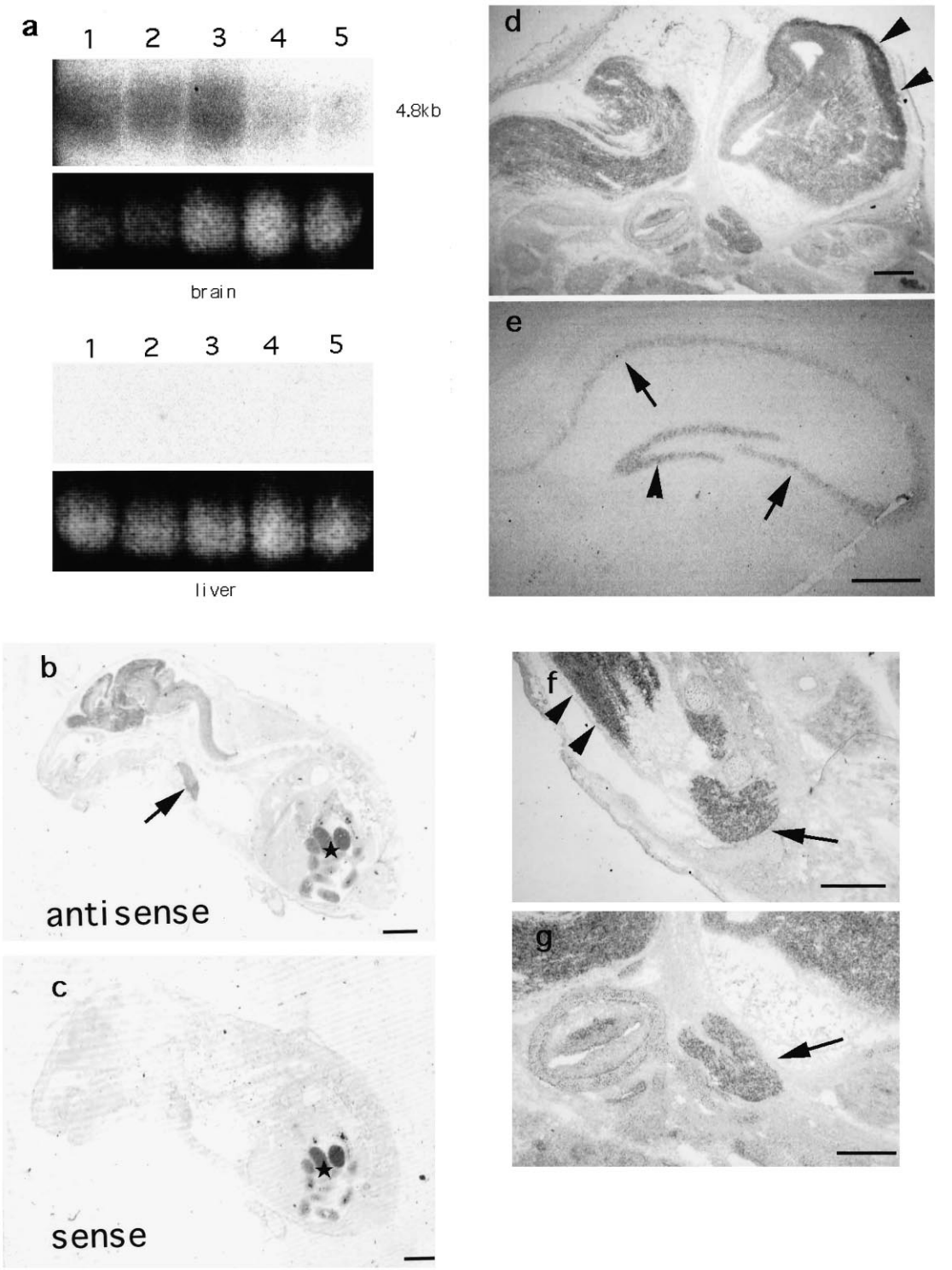


Fig. 1. Structure of the Eos gene product. Alignment of the predicted amino acid sequence of Eos with the sequences of Helios, Aiolos and Ikaros is shown. Amino acids outlined in black represent identical residues. Cysteine and histidine residues comprising the C2H2 type Zn finger domain are marked with closed triangles, except for the atypical cysteine residue in the fourth finger (indicated by an open triangle) and the histidine residues in the sixth finger (indicated by open squares).

and combined to yield a 2.8 kb cDNA that contained an open reading frame encoding a protein homologous to the Ikaros family (Ikaros, Aiolos and Helios, Fig. 1). We named this gene Eos to follow the naming of the family members. The Eos protein consisted of 533 amino acids and overall sequence similarities were as follows: Eos-Helios, 83.3%; Eos-Aiolos, 82.4%; Eos-Ikaros,72%. The Eos protein had several structural motifs common to the Ikaros family members, namely four Zn finger motifs in its N-terminal side, transcriptional activation domain and two Zn finger motifs implicated in homo- or hetero-dimerization with the family members. These domains had stronger homologies with corresponding domains of known members (Fig. 1) than with the remaining regions. The unusual cysteine residue in the fourth Zn finger (C2HC type) common to the Ikaros family members was also conserved in the Eos protein (Fig. 1, indicated by an open trian-



(Fig. 2.)

Fig. 2. Tissue expression of Eos mRNA. (a) Northern analysis of Eos mRNA expression in the developing brain and liver. 1: Embryonic day 15 (E15), 2: E18, 3: postnatal day 0 (P0), 4: P7, 5: adult. The upper panel showed a band of 4.8 kb in the developing brain. Note that the embryonic brain exhibited a strong signal for Eos mRNA and the intensity gradually decreased as the mouse grew. The lower panel showed that Eos mRNA expression was under the detectable level in the developing liver at all stages. In both panels, 18s ribosomal RNA bands stained with ethidium bromide showed that equal amounts of RNA were loaded in each lane. We examined the developing lung, kidney, thymus and spleen (P0–adult) similarly, but did not detect any signal for Eos mRNA (data not shown). (b) In situ hybridization analyses of Eos mRNA expression in the E18 embryo. Note that the brain and spinal cord were labelled intensely and ubiquitously by the Eos probe. The thymus was also weakly labelled (arrow). The gut (asterisk) exhibited staining but it was considered to be non-specific because similar staining was observed in the sense control (asterisk in c). (c) Sense control for in situ hybridization. An adjacent section to (b) was hybridized with the sense probe. Bars in b and c are 2 mm. (d) Eos mRNA expression in the E14 brain. The brain was labelled ubiquitously and the cortical plate (arrowheads) in which relatively differentiated neurons are localized was also intensely stained. Bar = 500 μm. (e) Eos mRNA expression in the P7 hippocampus. At this stage, the level of Eos mRNA expression was decreased and only the hippocampal pyramidal neurons (arrows) and granule neurons (arrowheads) were weakly positive. Bar = 250 μm. (f) Eos mRNA expression in the E14 dorsal root ganglion. The arrow indicates the dorsal root ganglion. Neurons were labelled intensely. The spinal cord was also positive (arrowheads). Bar = 300 μm. (g) Eos mRNA expression in the E14 trigeminal ganglion. The arrow indicates the trigeminal ganglion stained ubiquitously. Bar = 300 μm.
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gle). The sixth Zn finger motif (C2H2 type) also showed a characteristic structure in that five amino acids were inserted between histidine residues (Fig. 1, indicated by open squares), reminiscent of the zinc finger domain of the *Drosophila* hunchback protein [15].

We examined the tissue distribution of Eos mRNA by Northern blotting analysis. Eos transcript was detected predominantly in the brain as a band of 4.8 kb (Fig. 2a). The intensity of the signal was strong in the E15 and E18 brain and gradually decreased as the postnatal development proceeded (Fig. 2a, upper panel). No signals were detected in the liver, kidney, lung, thymus or spleen during all developmental stages we examined (Fig. 2a, lower panel for the liver, data not shown for the kidney, lung, thymus and spleen). The expression pattern was in marked contrast to those of the known Ikaros family members. Ikaros mRNA was primarily observed in the hemopoietic tissues in the embryo and later in the lymphoid tissues [1]. Aiolos and Helios mRNAs were also mainly expressed in the lymphoid lineage [6,7]. By in situ hybridization histochemistry, we detected a significant signal for Eos mRNA in the embryonic nervous system from the earliest age we examined (E11, data not shown). Its distribution was almost ubiquitous in the central (Fig. 2b and d) and peripheral nervous systems (Fig. 2f and g). Eos mRNA was weakly detected in the embryonic thymus (Fig. 2b, arrow), suggesting that Eos and Ikaros family members may be co-expressed in the thymus. There is, however, another possibility that Eos probe may have cross-hybridized with known members of the Ikaros family. Consistent with the Northern analysis, Eos mRNA expression decreased gradually after birth and at P7, only weak signals were observed in the pyramidal and granule cell layers of the hippocampus (Fig. 2e). In the adult, Eos mRNA was slightly observed in the hippocampus by in situ hybridization (data not shown).

Ikaros family members have been shown to interact with themselves and with other members through the C-terminal Zn finger domain [11]. As the overall structure of the predicted Eos protein was very similar to those of the known Ikaros family members, Eos protein was suspected to dimerize with itself and possibly with Ikaros family members. To test these possibilities, we employed the yeast two hybrid assay. As shown in Fig. 3, full length Eos protein (Eos-F) interacted with full length Ikaros protein (a) in yeast. However, Eos-N did not show any sign of interaction with Ikaros protein (b), while Eos-C did. These findings suggested that the Eos protein dimerizes through the C-terminal Zn finger domain with Ikaros protein, consistent with results of the previous studies

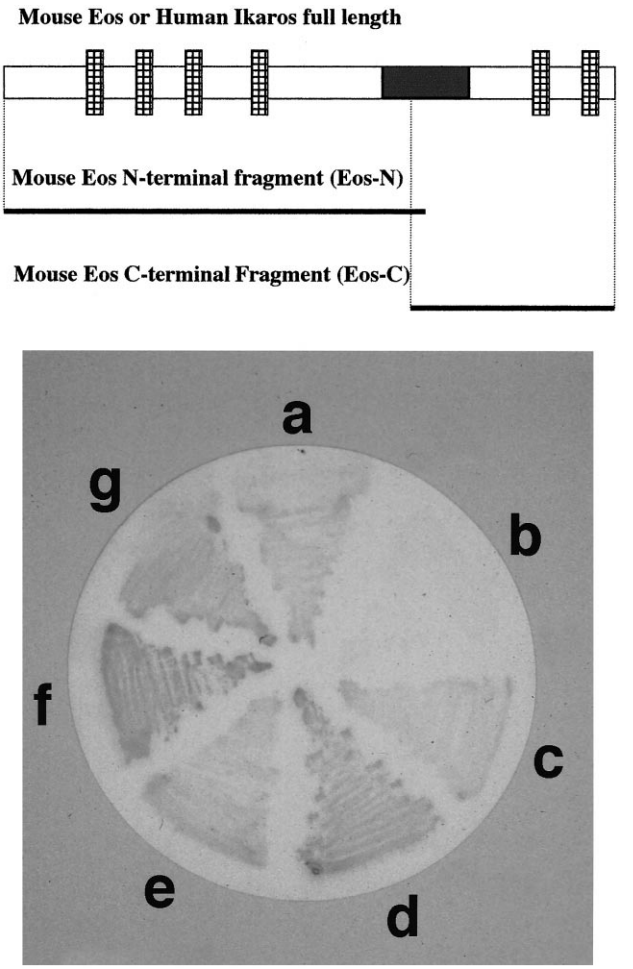

Fig. 3. Yeast two hybrid analyses of Eos and Ikaros protein interactions. The upper panel shows a schematic representation of the Ikaros family protein. The Zinc finger motif is depicted as a hatched box and the shaded box indicates the transcriptional activation domain. Relative positions of the mouse Eos-N and Eos-C are shown. The lower panel shows the results of yeast two hybrid analyses. a: Eos full length/Ikaros full length, b: Eos-N/Ikaros full length, c: Eos-C/Ikaros full length, d: Eos full length/Eos full length, e: Eos-N/Eos full length, f: Eos-C/Eos full length, g: positive control for protein-protein interaction. Plasmids (pVA3 and pTD1) that are known to interact in the yeast two hybrid system were transformed and a color reaction was performed.

(reviewed in [9,10]). Eos-F interacted with itself (d) and Eos-C (f) with a comparable color development. The combination of Eos-F and Eos-N showed a color reaction with less intensity but above background, suggesting that an interaction may take place (e). This interaction may be specific to the case of Eos-Eos self interaction as Eos-F did not appear to interact with Ikaros-N (b).

4. Discussion

The Ikaros family has been implicated in hemolymphoid development. Critical functions of this family in the lymphoid tissues were suggested by the loss of function experiments [2,16]. In the present study, we identified a novel member of this family, Eos, which is primarily expressed in the developing nervous system. By analogy, it is likely that Eos has important roles in the development of both central and peripheral nervous systems. Of the known members of this family, Ikaros mRNA expression was detected in the restricted region of the basal forebrain and midbrain of the embryo [1] and Helios mRNA was localized to the germinal zone of the embryonic brain [8]. It is possible that Ikaros and Helios exert their functions in these regions by dimerization with Eos. Judging from the distribution pattern, Eos probably has more essential functions in neural development than the other known family members. A recent report suggested that the neuronal enkephalin gene may be under control of Ikaros-like transcription factor(s) [17]. Eos may be involved in such determination of transmitter phenotype of the developing nervous system. Functional aspects of the Eos protein are now under investigation.

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