Bidirectional signalling through the EPH-family receptor Nuk and its transmembrane ligands

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RECEPTOR tyrosine kinases of the EPH class have been implicated in the control of axon guidance and fasciculation [1-7], in regulating cell migration [8], and in defining compartments in the developing embryo [9-11]. Efficient activation of EPH receptors generally requires that their ligands be anchored to the cell surface, either through a transmembrane (TM) region or a glycosyl phosphatidylinositol (GPI) group [12]. These observations have suggested that EPH receptors can transduce signals initiated by direct cell-cell interaction. Genetic analysis of Nuk, a murine EPH receptor that binds TM ligands, has raised the possibility that these ligands might themselves have a signalling function [6]. Consistent with this, the three known TM ligands have a highly conserved cytoplasmic region, with multiple potential sites for tyrosine phosphorylation [12-17]. Here we show that challenging cells that express the TM ligands Elk-L or Htk-L with the clustered ectodomain of Nuk induces phosphorylation of the ligands on tyrosine, a process that can be mimicked both *in vitro* and *in vivo* by an activated Src tyrosine kinase. Co-culture of cells expressing a TM ligand with cells expressing Nuk leads to tyrosine phosphorylation of both the ligand and Nuk. These results suggest that the TM ligands are associated with a tyrosine kinase, and are inducibly phosphorylated upon binding Nuk, in a fashion reminiscent of cytokine receptors [18]. Furthermore, we show that TM ligands, as well as Nuk, are phosphorylated on tyrosine in mouse embryos, indicating that this is a physiological process. EPH receptors and their TM ligands therefore mediate bidirectional cell signalling.

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Nuk belongs to a subclass of EPH receptors that bind specifically to the TM subgroup of EPH-receptor ligands [11,14,19]. Genetic analysis of *Nuk* has revealed a physiological requirement for this receptor for correct pathfinding of specific anterior commissure (AC) axons in the mouse brain [6]. Surprisingly, however, a truncated Nuk polypeptide containing the extracellular and TM domains, but lacking the kinase domain, supports normal AC formation in some genetic backgrounds. Nuk is primarily expressed in cells immediately ventral to the AC, whereas TM ligands are expressed in the commissural axons, raising the possibility that the TM ligands might themselves possess a signalling function, which is activated by binding of the Nuk extracellular domain [6]. The three known TM ligands have highly conserved cytoplasmic domains, and are virtually identical over their C-terminal 33 amino acids [12-17]. These sequences contain five potential tyrosine-

phosphorylation sites [20] Figure 1(*a*), suggesting that the TM ligands might signal in conjunction with a tyrosine kinase. Consistent with this, a glutathione S-transferase (GST) fusion protein containing the cytoplasmic domain of human Elk-L (residues 262-343) was tyrosine-phosphorylated by v-Src *in vitro*, whereas GST alone, or a fusion protein containing the Elk-L extracellular domain, was not (Figure 1(*b*), and data not shown).

To investigate whether full-length Elk-L or Htk-L could be phosphorylated on tyrosine *in vivo*, these TM ligands were expressed in Cos-1 cells either alone or in combination with v-Src. The ligands were then precipitated from the transfected cells using either an antibody against the common C-terminal region of Elk-L and Htk-L (anti-ligand) or a fusion protein containing the extracellular domain of Nuk fused to the Fc region of the immunoglobulin heavy chain (Nuk-Fc [11]), which binds with high affinity to the extracellular domain of TM ligands. When these precipitates were blotted with ligand antibody, a diffuse band corresponding to a relative molecular mass (M_r) of approximately 45K-48K was specifically detected in cells transfected with Elk-L, whereas a protein of M_r between 38K and 46K was identified in cells transfected with Htk-L (Figure 1(*c*), lower panel). The predicted M_r for Elk-L is 38K and that for Htk-L is 37K, and their slow electrophoretic mobility is apparently due to glycosylation. Immunoblotting of anti-ligand immunoprecipitates from transfected Cos-1 cells with antibodies against phosphotyrosine (Figure 1(*c*), upper panel) showed that both Elk-L and Htk-L were basally phosphorylated on tyrosine at low levels. Co-transfection of Elk-L with v-*src* led to the appearance of a highly tyrosine-phosphorylated approximately 50K form of Elk-L in both anti-ligand and Nuk-Fc precipitates (Figure 1(*c*), upper panel, and Figure 1(*d*)). In addition, a tyrosine-phosphorylated protein of 130K-140K co-precipitated with Elk-L from cells coexpressing ligand and v-Src. v-Src also induced strong tyrosine phosphorylation of Htk-L, which migrated as a broad band of 38K-48K in the phosphorylated form. Immunoprecipitation of both Elk-L/Htk-L and the 130K protein was markedly reduced by addition of the immunizing peptide, which competes for antibody binding (data not shown). In v-*src* co-transfected cells, there was a reduction in the total amount of either ligand detected in western blots by anti-ligand antibodies. These antibodies, which were raised against the C-terminal part of Elk-L, may be less efficient in recognizing highly tyrosine-phosphorylated forms of the denatured ligands in an immunoblot. These results show that Elk-L and Htk-L are potent *in vivo* substrates for an activated Src tyrosine kinase and can be detected in association with other phosphotyrosine-containing proteins in cells expressing both ligand and v-Src.

FIG. 1 Phosphorylation of transmembrane ligands by v-Src. *a*, Alignment of human Elk-L, Htk-L and Elk-L3 cytoplasmic domains. Black and grey boxes indicate residues conserved in all or two TM-ligands, respectively; asterisk indicates conserved tyrosines. Elk-L is also referred to as Lerk-2 [13] and Cek5-L [14], Htk-L as Lerk-5 [26] and Elf-2 [15] and Elk-L3 as NLerk-2 [29]. *b*, Phosphorylation of GST-hElk-L cytoplasmic-domain fusion protein (GST-Elk-L cyt) *in vitro* by v-Src. v-Src was immunoprecipitated from v-src-transformed Rat-2 cells and incubated with GST fusion proteins in the presence of [γ-^{32}P]ATP. *c*, *d*, Tyrosine phosphorylation of Elk-L and Htk-L upon coexpression with v-Src in Cos-1 cells. Ligands were immunoprecipitated with anti-ligand antibodies (*c*) or using Nuk-Fc as an affinity reagent (*d*), and immunoblotted with anti-phosphotyrosine antibodies. The band at approximately 100K in d represents cross-reaction of Nuk-Fc with protein A-HRP.
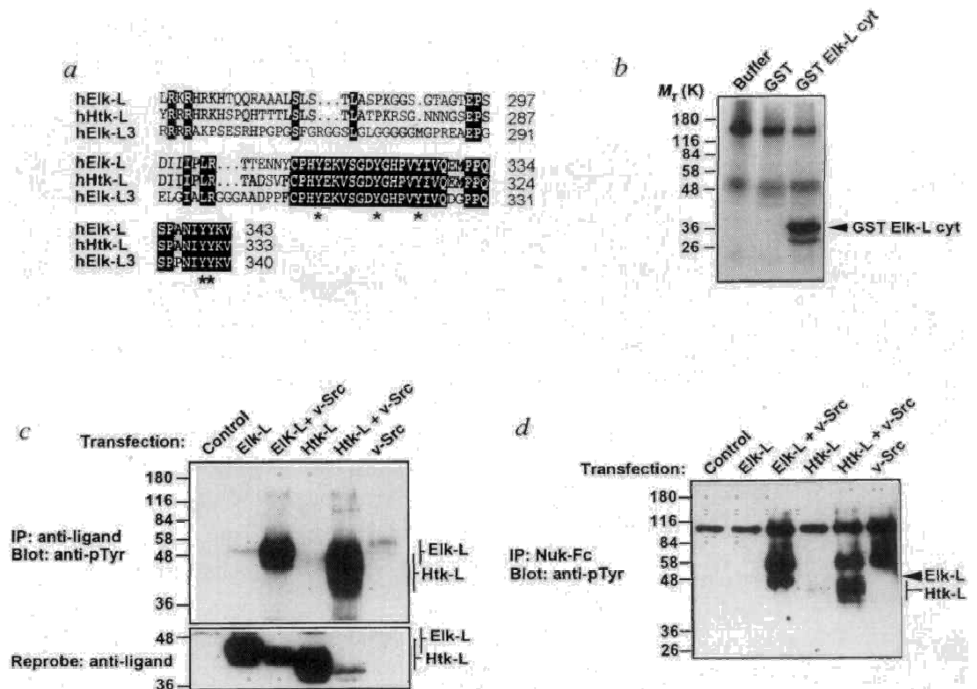
The phosphorylation of TM ligands on tyrosine may provide a mechanism by which signals are transmitted into ligand-presenting cells. We reasoned that such a signal might be activated by the clustering of ligands on the cell surface. We therefore exposed Cos-1 cells expressing Elk-L or Htk-L to the Nuk extracellular domain, in the form of Nuk-Fc fusion protein clustered with anti-immunoglobulin. Under these conditions, Nuk-Fc induced a several-fold increase in tyrosine phosphorylation of both Elk-L and Htk-L, whereas no stimulation of ligand tyrosine phosphorylation was induced by Fc alone Figure 2(*a*). The tyrosine-phosphorylated band immunoprecipitated by the anti-ligand serum was markedly reduced by addition of excess ligand C-terminal peptide to the immunoprecipitates. This experiment indicates that binding of clustered Nuk-Fc to the TM ligands activates an endogenous tyrosine kinase in Cos-1 cells which can subsequently phosphorylate Elk-L and Htk-L. These results were obtained using exogenously overexpressed ligand, however. To corroborate our observations in a physiologically relevant cell type, we used the human neuroepithelioma cell line CHP-100, which expresses endogenous Elk-L [12]. Incubation of CHP-100 cells with clustered Nuk-Fc led to a striking increase in tyrosine phosphorylation of Elk-L and to co-

phosphorylation of Elk-L or Htk-L, consistent with stimulation of a ligand-associated tyrosine kinase in the ligand-expressing cells Figure 2(*c*). Parental NG108 cells lacking Nuk were without effect and conversely, no phosphorylation of either TM ligands or Nuk was induced using untransfected Cos-1 cells (Figure 2(*c*), and data not shown).

precipitation of several tyrosine-phosphorylated polypeptides Figure 2(*b*). Thus, the binding of the Nuk extracellular domain to a cell that normally expresses Elk-L also leads to tyrosine kinase activation and concomitant Elk-L phosphorylation.

These findings raised the possibility that interaction of a cell expressing TM ligands on its surface with a second cell expressing Nuk might lead to the activation of the Nuk receptor, and subsequent signalling within the Nuk-expressing cell, and also to the activation of a ligand-associated kinase and consequent ligand phosphorylation. We therefore co-cultured Cos-1 cells expressing Elk-L or Htk-L with the neuronal cell line NG108-15 [21] (NG108), which lacks endogenous EPH receptors that bind TM ligands, or with a transfected NG108 clone that stably expresses high levels of the 130K mouse Nuk protein (NG108-Nuk). In co-cultures of ligand-expressing cells with NG108-Nuk cells, we observed Nuk tyrosine phosphorylation, reflecting activation of the Nuk catalytic domain, and also tyrosine
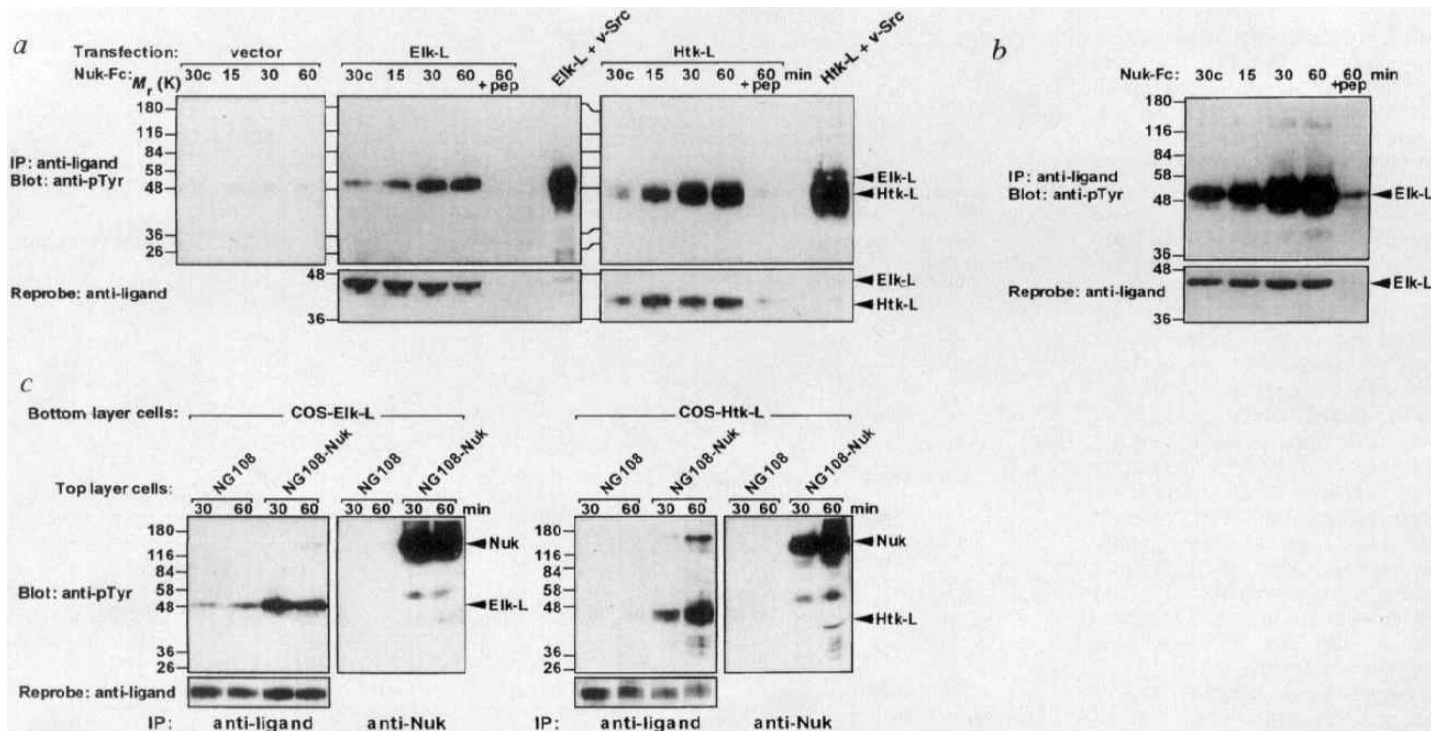
FIG. 2 Stimulation of tyrosine phosphorylation of TM ligands by Nuk extracellular domain and Nuk-expressing cells. *a*, Cos-1 cells were transiently transfected with Elk-L, Htk-L, or control expression vectors, and treated with 2 μg ml⁻¹ clustered Nuk-Fc or Fc (c) for the indicated periods (min). Ligands were immunoprecipitated (IP) with anti-ligand antibodies and blotted with anti-phosphotyrosine antibodies (top panels). Lower panels, anti-ligand blot (reprobe). '+pep' Competing immunizing peptide was added. *b*, CHP-100 cells, which express endogenous Elk-L, were stimulated and immunoblotted as for a. Top panel, anti-phosphotyrosine blot; lower panel, anti-ligand blot (reprobe). *c*, Bidirectional signalling between Nuk-expressing and TM-ligand-expressing cells in co-culture. Cos-1 cells transiently transfected with Elk-L or Htk-L were co-cultured with parental or Nuk-expressing NG108 cells for the indicated times. Left panels, anti-ligand immunoprecipitate; right panels, anti-Nuk IP from pooled, co-cultured cells. Top, anti-phosphotyrosine blots; bottom, anti-ligand blots (reprobe).

The observation that Elk-L and Htk-L are inducibly phosphorylated on tyrosine in cultured cells on exposure to clustered Nuk-Fc or Nuk-expressing cells suggests that this may be a physiological event. We therefore immunoprecipitated protein lysates from mouse embryos at
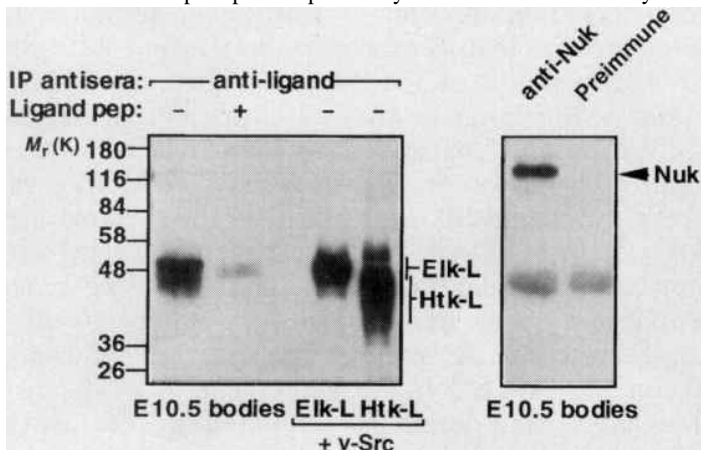

FIG. 3 Transmembrane ligands and Nuk are phosphorylated on tyrosine in the mouse embryo. Anti-phosphotyrosine immunoblot of TM ligands (left) and Nuk receptor (right) immunoprecipitated from E10.5 mouse body tissue. Lysed tissue was immunoprecipitated with anti-Nuk or pre-immune serum, or anti-ligand antibodies with or without addition of excess competing immunizing peptide (ligand pep). Results for E10.5 head tissue were essentially identical. The mobility of tyrosine-phosphorylated Elk-L and Htk-L is indicated by inclusion of v-Src-phosphorylated TM ligands on the gel (Elk-L + v-Src and Htk-L + v-Src).

10.5 days of development (E10.5) with antibodies against either TM ligands or Nuk, and immunoblotted the immune complexes with antibodies against phosphotyrosine Figure 3. Nuk immunoprecipitated from embryonic body or head tissue was phosphorylated on tyrosine (Figure 3, and data not shown). Furthermore, anti-ligand antibodies specifically precipitated phosphotyrosine-containing polypeptides from these embryonic lysates that co-migrated with v-Src-phosphorylated TM ligands. The intensity of the tyrosine-phosphorylated band immunoprecipitated by the anti-ligand antibodies was markedly reduced by addition of excess ligand C-terminal peptide. These results show that it is not only EPH receptors such as Nuk but also their TM ligands that

are phosphorylated on tyrosine in the developing mouse embryo.

EPH receptors have been implicated in establishing boundaries between two distinct cell types, for example in the rhombomeres of the hindbrain and in development of the forebrain [9-11]. It would therefore be advantageous if cell-cell contact initiated a bidirectional signal thereby regulating the phenotype of both and ligand-expressing cells. We have proposed a biochemical mechanism for achieving such bidirectional signalling Figure 4. In the neuronal cell line NG108, activation of Nuk by TM ligands leads not only to Nuk autophosphorylation, but also to phosphorylation of potential receptor targets (Figure 2(c), and S.J.H. et al., manuscript in preparation). Our data also indicate that binding of Nuk to TM ligands activates a tyrosine kinase in the ligand-expressing cell, leading to phosphorylation of the conserved C-terminal region of the ligand itself. One scheme consistent with our results is that phosphorylation of the ligands by Src-like

FIG. 4 Proposed model for bidirectional signalling by Nuk and its TM ligands. Interaction of the ligand-expressing cell (right) with the receptor-expressing cell (left) promotes aggregation and autophosphorylation of the receptor. This is followed by recruitment of SH2-domain-containing proteins to phosphorylated tyrosines, for example in the juxtamembrane region [27,28], and tyrosine-phosphorylation of cellular proteins. Concomitantly, interaction of the receptor with TM ligands causes ligand clustering and phosphorylation by an associated tyrosine kinase, possibly leading to propagation of signals in the ligand-presenting cell.
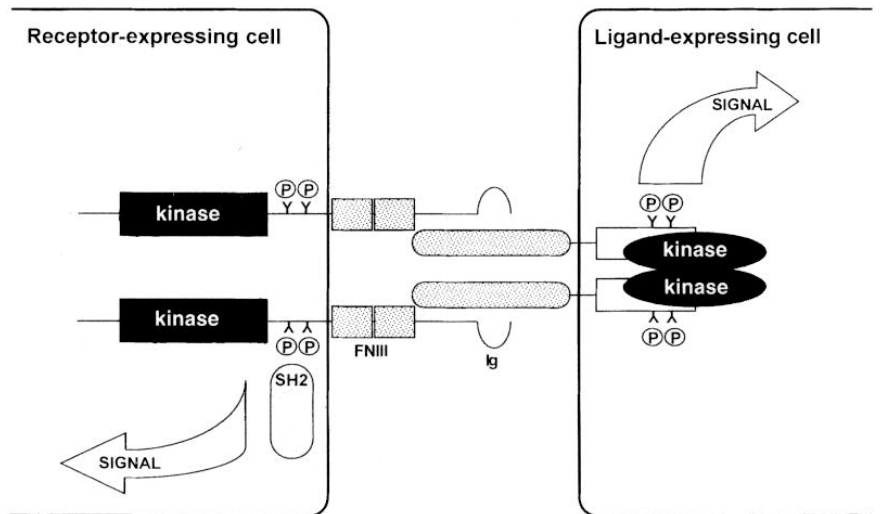
kinases induces the binding of SH2-containing proteins which then transmit signals within the ligand-expressing cell; alternatively, ligand phosphorylation might modulate interactions with the cytoskeleton. However, the identities of the tyrosine kinases which phosphorylate TM ligands *in vivo* and the signalling proteins which bind activated ligands are not known. EPH family tyrosine kinases therefore function not only as receptors but also as activating ligands for transmembrane proteins such as Elk-L, Htk-L and presumably the recently cloned Elk-L3 [17]. Interactions of this type may represent a general and economic mechanism by which cellular behaviour is controlled during embryonic development and axonal migration [22-24].

Methods

Reagents. Anti-ligand antibodies (Santa Cruz) were raised against residues 326-343 of hElk-L, and also recognize Htk-L. Anti-Nuk serum was described previously [2]. Monoclonal anti-v-Src antibodies were from Oncogene Science. GST-Elk-L cyt contains the whole cytoplasmic domain (residues 262-343) of hElk-L (ref. 12) cloned into pGEX4T2.

Transfection and cell stimulation. Cos-1 cells were transiently transfected with 5-10 µg DNA using lipofectin reagent (Gibco). Cells were collected approximately 60 h post-transfection after 20 h in medium containing 0.5% fetal bovine serum. Human neuroepithelial CHP-100 cells were serum-starved for 8 h before stimulation. Nuk-Fc [11] and Fc were clustered using anti-human IgG (Jackson Immunoresearch) for 1-2 h at 4 °C and diluted to 2 µg ml^{-1} in serum-free medium before applying to cells.

Co-cultures. NG108-15 cells (NG108: mouse neuroblastoma × rat glioma fusion [21]) were stably transfected with an expression vector containing full-length Nuk, and individual G418-resistant cloes isolated (NG108-Nuk cells). Parental or Nuk-expressing NG108 cells were removed from the plate by tituration and resuspended in PBS plus magnesium and calcium. Cell suspensions were placed on top of serum-starved Cos-1 cells transiently expressing TM ligands and co-cultured for 30 or 60 min at 37 °C with 5% CO_2. Pooled cells were lysed and lysates divided into two for immunoprecipitation.

Immunoprecipitation and immunoblotting. Cells were lysed in PLC lysis buffer [2]. Lysates of E10.5 embryos from natural matings were prepared as described [2] and precleared by incubation with protein A-Sepharose. Immunoprecipitations were done using 1 µg anti-ligand antibodies, 5 µl anti-Nuk serum or 10 µg of Nuk-Fc fusion protein with protein A-Sepharose, or 1 µg monoclonal anti-v-Src antibodies with anti-mouse agarose for 1-2 h, then washed three times with HNTG buffer [2]. Protein complexes were separated by 10% SDS-PAGE transferred to an Immobilon-P membrane (Millipore) and immunoblotted. Anti-phosphotyrosine blots were performed using 4G10 (UBI), except for Figure 1(*d*), for which polyclonal anti-phosphotyrosine antibodies were used. Membranes were stripped with 0.1 M glycine, pH 2.5, where indicated. Peptide competition with immunizing peptide (Santa Cruz) used approximately 100-fold excess peptide.

***In vitro* kinase assay.** v-Src was immunoprecipitated from v-src-transformed Rat-2 cells and incubated for 10 min at room temperature with 5 µCi of [γ-^{32}P]ATP in Src-kinase reaction buffer [25], or buffer containing 10 µg purified GST or GST-Elk-L cyt as exogenous substrate. Products were separated and ^{32}P-labelled proteins detected by autoradiography.

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