Canoe functions at the CNS midline glia in a complex with Shotgun and Wrapper-Nrx-IV during neuron-glia interactions

Jana Slováková and Ana Carmena*

SUMMARY
Vertebrates and insects alike use glial cells as intermediate targets to guide growing axons. Similar to vertebrate oligodendrocytes, *Drosophila* midline glia ensheathe and separate axonal commissures. Neuron-glia interactions are crucial during these events, although the proteins involved remain largely unknown. Here, we show that Canoe (Cno), the *Drosophila* ortholog of AF-6, and the DE-cadherin Shotgun (Shg) are highly restricted to the interface between midline glia and commissural axons. *cno* mutant analysis, genetic interactions and co-immunoprecipitation assays unveil Cno function as a novel regulator of neuron-glia interactions, forming a complex with Shg, Wrapper and Neurexin IV, the homolog of vertebrate Caspr/paranodin. Our results also support additional functions of Cno, independent of adherens junctions, as a regulator of adhesion and signaling events in non-epithelial tissues.

KEY WORDS: Neuron-glia interactions, Canoe/AF-6, *Drosophila*, CNS, Wrapper, DE-cadherin, Nrx-IV, Midline

INTRODUCTION
Intricate and reciprocal neuron-glial interactions are essential for proper nervous system development across species (Crews, 2010; Klambt, 2009; Lemke, 2001). Early on during neuronal development, glial cells act as guidepost cells, i.e. intermediate targets for growing pioneer axons on the way to their final destination (Bastiani and Goodman, 1986; Bentley and Caudy, 1983; Learte and Hidalgo, 2007). Neurons, in turn, are essential for glia migration, proliferation and survival (Birchmeier and Nave, 2008; Brinkmann et al., 2008; Klambt, 2009). Whereas vertebrate glial cells, oligodendrocytes in the central nervous system (CNS) and Schwann cells in the peripheral nervous system (PNS), generate myelin sheaths around nerves to isolate and protect axonal tracts, many invertebrates, such as *Drosophila melanogaster*, do not produce myelin. However, *Drosophila* CNS midline glial cells ensheathe and separate axonal commissures in a process in which neuron-glial interactions are crucial. Hence, *Drosophila* midline glia constitute an amenable model system for untangling the complex and still largely unknown molecular mechanisms that underlie neuron-glial interactions. This, in turn, is essential for our in-depth understanding of the myelination process in development and disease (Bhat, 2003; Crews, 2010; Edenfeld et al., 2005; Jacobs, 2000; Sherman and Brophy, 2005).

*Drosophila* CNS midline cells are extremely well characterized in terms of both their location and molecular markers. In a mature CNS, ~22 midline cells are present per segment, three of which constitute the midline glia (MG) (Beckervordersandforth et al., 2008; Bossing and Technau, 1994; Jacobs, 2000; Klambt et al., 1991; Wheeler et al., 2006). At early stages, two types of MG have been molecularly characterized: anterior (six cells) and posterior (four cells) (Kearney et al., 2004). At later stages, all posterior MG (PMG) die and only three of the six anterior MG (AMG) survive (Wheeler et al., 2009); these are the three AMG present per segment in a mature CNS (stage 17), as mentioned above. One of the main functions of the MG is to provide the guidance cues required for proper axon pathfinding at the midline (Harris et al., 1996; Kidd et al., 1999; Mitchell et al., 1996). Another crucial function of the *Drosophila* MG is to separate and ensheathe the anterior and posterior commissures present per segment. Neuron-glial intercellular communication is essential during these processes, although the molecules involved remain largely uncharacterized (Edenfeld et al., 2005; Jacobs, 2000). Recently, the transmembrane protein Neurexin IV (Nrx-IV), which is the *Drosophila* ortholog of vertebrate Caspr/paranodin and is present in axonal membranes, has been shown to interact physically with the Immunoglobulin (Ig) superfamily member Wrapper, a glycosphatidylinositol (GPI)-linked protein expressed on the surface of the MG. Moreover, this interaction is fundamental for neuron-glial adhesion and, consequently, for the correct ensheathment of commissural axon fascicles (Banerjee et al., 2006; Baumgartner et al., 1996; Noordermeer et al., 1998; Stork et al., 2009; Wheeler et al., 2009).

Here, we show that the PDZ (PSD-95, Discs large, ZO-1) domain-containing protein Canoe (Cno) (Miyamoto et al., 1995) and the DE-cadherin Shotgun (Shg) (Tepass et al., 1996) are expressed in the MG, where they are highly concentrated at the interface between MG and commissural axons. *cno* loss-of-function mutant embryos showed clear defects in MG migration as well as in commissural axon ensheathment and subdivision. Indeed, Cno colocalized with Nrx-IV and Wrapper at the axon-glial interface and interacted genetically with them during this process. *shg* mutant embryos also displayed strong defects in MG enwrapping of commissural axons and a failure in Cno subcellular localization. Moreover, Wrapper formed a complex in vivo with both Shg and Cno. We propose that Cno and Shg in the MG link with the Wrapper-Nrx-IV complex and act as novel functional players that are crucial for regulating neuron-glial interactions at the midline.
MATERIALS AND METHODS

Drosophila strains and genetics

The following mutant stocks were used (all from the Bloomington Stock Center unless stated otherwise): cno\(^+\), cno\(^{mis10}\) (Miyamoto et al., 1995), Df(3R)ED5147 (Drosophila Genetic Resource Center, Kyoto, Japan), wrapper\(^{173}\), shg\(^{e}\), Nrx-IV\(^{304}\), Nrx-IV-GFP (CA06597) (Morin et al., 2001), sim-Gal4, slit-Gal4 (Scholz et al., 1997), elav-Gal4, maternal-Gal4 [V32 (Speicher et al., 2008)], eagle-Gal4, Df(3L) Exel 6116 and UAS-Nrx-IV-Exon4 (Stork et al., 2009) and UAS-mCD8::GFP, UAS-cno (Carmena et al., 2006). The crosses GAL4\(\times\)UAS were carried out at 25°C and 29°C, yw was used as the reference control wild-type strain. Balancer chromosomes containing different lacZ or GFP transgenes were used for identification of homozygous mutant embryos.

Immunohistochemistry, immunofluorescence and microscopy

Embryo fixation and antibody staining were carried out by standard protocols except as specified below. The following primary antibodies were used: rabbit anti-Cno 1/400 (Speicher et al., 2008); mouse BP102 1/100 [Developmental Studies Hybridoma Bank (DSHB)]; C555.6 mouse anti-Slit 1/100 (DSHB); 10D3 mouse anti-Wrapper 1/50-1/200 (DSHB); rat anti-DE-cadherin 1/20 (DSHB); rat anti-Elav 1/400 (DSHB); rabbit anti-HRP 1/5000 (Jackson); goat anti-HRP 1/100-1/500 (Jackson); rabbit anti-β-galactosidase 1/100,000 (Cappel); and mouse anti-β-galactosidase 1/8000 (Promega). Secondary antibodies coupled to biotin (Vector Labs), Slit 1/100 (DSHB); 10D3 mouse anti-Wrapper 1/50-1/200 (DSHB); rat anti-Cno 1/400 (Speicher et al., 2008); mouse BP102 1/100 and rabbit anti-Cno 1/400 (Speicher et al., 2008) were used for immunostaining with the anti-Cno antibody, embryos were fixed using the protocol described above. All micrographs shown in figures represent single sections immunostained with the anti-Cno antibody, embryos were fixed using the protocol described above. All micrographs shown in figures represent single sections immunostained with the anti-Cno antibody, embryos were fixed using the protocol described above.

RESULTS

Cno is localized at the neuron-glia interface in the midline

We had observed that Cno is expressed in the midline during the differentiation of the CNS (our unpublished observations). To investigate a potential function of Cno in this process, we analyzed its midline expression in detail. Cno was first detected at stage 12, at the sites of contact between the MG and the ectoderm and at some locations in the AMG (Fig. 1A-A\(^{\prime}\)). Later, during stage 12 [12/3; stage according to Klümbt et al. (Klümbt et al., 1991); see also Wheeler et al. (Wheeler et al., 2009)], Cno was mainly detected in the PMG (Fig. 1B-B\(^{\prime}\)). This expression persisted as far as stage 14. At this point, the AMG have already enwrapped the anterior commissure and Cno was present at the interface between the AMG and the anterior commissural axons (Fig. 1C-C\(^{\prime}\)). By stage 15 Cno was highly concentrated at the interface between the MG and the two commissures (Fig. 1D-D\(^{\prime}\)). At late stages (stage 17) only AMG are present and the three AMG extend processes that further subdivide the commissures into distinct axon bundles. Cno was highly restricted to these processes at this stage, colocalizing with the glial marker Slit, which itself is more widely distributed in the MG (Fig. 1E,E\(^{\prime}\)). In a ventral view at stage 17, Cno was detected in the MG, along with Slit, in close contact with anterior and posterior commissural axons (Fig. 1F-F\(^{\prime}\)). Therefore, Cno is present in the MG throughout the process of MG migration, commissural axon enwrapping and subdivision.

Cno is necessary for MG migration and for commissural axon enwrapping and subdivision

Given the characteristic expression of Cno in the MG from stage 12, we analyzed a potential requirement of Cno during MG migration and commissural axon enwrapping. In cno\(^{−}\) null mutant embryos, striking defects in these processes were observed in 55.2% of the segments analyzed throughout stages 15-17 (n=181) (Fig. 2A-G). Indeed, anterior and posterior commissures were hardly separated from each other (Fig. 2C,C\(^{\prime}\),F-G) or they displayed an abnormal, wider morphology (not shown). Likewise, the projections that the AMG normally send into the commissures at stage 17 to enwrap individual axons were not formed in 50% of cno\(^{−}\) mutant defective segments analyzed at this stage (n=72) (Fig. 2B,E,G). All these phenotypes were also observed with a similar expressivity and penetrance in cno\(^{−}\) over the Df(3R)ED5147, a deficiency that eliminates cno, and over cno\(^{mis10}\); another cno null allele (Miyamoto et al., 1995) (data not shown). cno gain-of-function at the MG (slit-Gal4>UAS-cno) also caused defects (10.3%, n=87 segments at 25°C; 11.1%, n=81 at 29°C), as revealed by Wraper expression as a MG marker. Specifically, the MG of adjacent segments were very frequently in contact as if cell adhesion was enhanced. The same phenotype was observed at slightly higher penetrance when two copies of cno were used (17.5%, n=126 segments at 25°C (Fig. 2H,I)). The overexpression of Cno using the neural-specific driver elav-Gal4 did not cause any apparent phenotype in the MG (1.4%, n=70 segments at 25°C). Intriguingly, the cno\(^{−}\)/cno\(^{−}\) mutant phenotype was partially rescued when Cno was specifically overexpressed in the MG under the slit-Gal4 (11% of defective segments at stage 16-17, n=109, compared with 50.0% defective segments, n=75, in cno\(^{−}\)/cno\(^{−}\) mutant embryos at this stage; see also above). These results strongly support a function of Cno during MG migration, commissural axon enwrapping and subdivision.

Cno colocalizes and genetically interacts with Wraper and Nrx-IV

The Cno superfamily protein Wraper is expressed in the MG and is crucial to properly ensheath commissural axons (Noordermeer et al., 1998). Very recently, two studies have shown how Wraper acts through the transmembrane protein Nrx-IV, which is expressed mainly on neuronal membranes and it is highly enriched at the interface between neuronal surfaces and the MG. This interaction is fundamental for neuron-glia adhesion and, consequently, for the correct enwrapping of commissural axons fascicles (Banerjee et al., 2006; Baumgartner et al., 1996; Stork et al., 2009; Wheeler et al., 2009). Given that the midline phenotype of the cno\(^{−}\) mutants that we observed was very similar to that described for Nrx-IV and wraper mutants (Stork et al., 2009; Wheeler et al., 2009), we analyzed functional relationships between cno, wraper and Nrx-IV. First, double immunostaining showed that Cno colocalizes with Wraper (Fig. 3A-B\(^{\prime}\)) and Nrx-IV (Fig. 3C,C\(^{\prime}\)) at the MG. Then, we studied the phenotype of double heterozygotes to detect
Fig. 1. Cno is expressed in the midline glia. (A–F) Cno expression in the midline glia (MG) throughout embryogenesis of *sim*-Gal4 UAS-CD8::GFP Drosophila embryos. Composite confocal images of single CNS segments in sagittal (sag; A–D) or ventral (ven; E–F) views. A lower magnification overview of the CNS, including several segments, is shown for each stage (A–D,E,F). Anterior is left. Stage and orientation are shown in the upper right corner. mn, midline neurons; ac, anterior commissure; pc, posterior commissure; AMG, anterior midline glia; PMG, posterior midline glia. (A–B) Cno (magenta) is first detected in the MG (arrows) at stage 12 (BP102, a general axonal marker, is in blue). (C,C′) At stage 14, when AMG has already enwrapped the anterior commissure, Cno is detected at the interface between AMG and this commissure (arrows in the AMG). Cno is also detected at the PMG (arrow). (D,D′) By stage 15, when both commissures have clearly separated, Cno is concentrated at the sites of close contact between MG and commissures (arrows). (E–F) At stage 17, Cno is highly restricted to the cytoplasmic projections that MG extend to further subdivide commissural axons into distinct axon bundles (E–F). A ventral view of a stage 17 embryo is shown (F–F′). Slit (labeling the MG) is in blue. (A′, B′, C′, D′, E′) Schematics representing midline cells (including MG and mn) in a sagittal single segment illustrating the changes that occur at the stages shown in the corresponding confocal micrographs. For clarity, only one cell of the AMG and PMG is shown (see text for details). Scale bars: 10 μm.
possible functional interactions between these proteins. Nrx-IV^304, +/+, cno^2 transheterozygotes showed specific defects in commissural axon ensheathment and separation in a significant number of segments (35.6%, n=177) (Fig. 4A,A’,C). wrapper^175/+; cno^2/+ embryos showed 10.5% defective segments (n=95). In addition, the penetrance of the cno^2/cno^2 phenotype was significantly enhanced (P=0.004) in a sensitized background in which the dose of wrapper was reduced (i.e. in wrapper^175/+; cno^2/cno^2 mutant embryos) (Fig. 4C). The expressivity of the wrapper^175/+; cno^2/cno^2 phenotype was also much higher than that of the cno^2/cno^2 phenotype (in 71% of the wrapper^175/+; cno^2/cno^2 segments with a phenotype, this was stronger than the phenotype found in cno^2/cno^2 mutants; Fig. 4B,B’). Taking all these data into account, our results support a functional relationship between Cno and Nrx-IV/Wrapper at the midline of the CNS during neuron-glia interactions.
Cno forms a complex in vivo with Wrapper and Nrx-IV

The functional relationships between Cno, Wrapper and Nrx-IV, as well as their colocalization, being highly restricted to the MG/commissural axon contacts, prompted us to investigate whether Cno forms a complex with Wrapper and Nrx-IV. Co-IP experiments from embryo extracts confirmed the presence of Cno-Wrapper and Cno-Nrx-IV aggregates in vivo (Fig. 5). We then investigated whether the localization of Cno was altered in wrapper mutants. In both Nrx-IV and wrapper mutants, the localization of Cno was affected, being completely or partially missing from the commissures and frequently located between them (Fig. 6A-C). When Nrx-IV was expressed in a subset of commissural neurons (i.e. eagle-Gal4>>UAS-Nrx-IV) in an Nrx-IV mutant background, the localization of Cno, along with that of Wrapper (MG), was partially rescued (see Fig. S1 in the supplementary material) (Stork et al., 2009). We next examined whether the defects in Cno localization found in Nrx-IV and wrapper mutants were a secondary effect of the failure in MG migration and enwrapping of commissural axons found in these mutants. We used Slit as a MG marker along with Cno and analyzed higher magnifications of Nrx-IV and wrapper mutants in sagittal views (Fig. 6D-F). We found that failure in Cno localization (including the absence of Cno at MG projections) perfectly correlated with the loss of MG in these mutants (Fig. 6D-F). These results further support a requirement for Cno at the MG, not at the commissural axons. However, the subcellular localization of Cno in the remaining MG found in Nrx-IV and wrapper mutants was unaltered: Cno was still highly restricted to the interface between MG and commissural axons (Fig. 6E-F’). This suggests that other protein(s) stabilize Cno at this location (see below).

Shotgun localizes and functions at the MG in a complex with Wrapper

Our results indicate that Cno at the MG forms a complex in vivo with Wrapper and Nrx-IV. But, how could the cytoplasmic protein Cno be interacting at the MG with Wrapper, a GPI-linked protein that lacks a cytosolic domain? Additionally, why is Cno still highly localized at the MG and was highly enriched, as is Cno, at the interface between MG and commissural axons (Fig. 7A-C). Moreover, shg mutant embryos showed strong defects in the MG in 46.5% of the segments analyzed (n=88), with commissural axons frequently fused (61%; Fig. 7D,E), MG missing (14.6%; Fig. 7D,E) or MG misplaced (85.4%; Fig. 7E,G, compare with 7D,F).
Intriguingly, the subcellular localization of Cno at the MG was altered in shg mutants (Fig. 7H-I). Moreover, Shg formed a complex in vivo with Wrapper (Fig. 7J). Taking all these data into account, we propose that Shg functions at the MG, where it acts as a linker between Cno and the Wrapper-Nrx-IV complex (Fig. 7K).

**DISCUSSION**

The midline constitutes a key boundary of bilateral organisms. In vertebrates, it is the floorplate and the functionally equivalent structure in *Drosophila* is the mesectoderm, which gives rise to all midline cells, neurons and glia, in the most ventral part of the embryo. MG are of great relevance at the midline as an intermediate target during axonal pathfinding, providing both attractive and repulsive guidance cues. These signals allow contralateral axons to cross the midline but never to recross, and they also keep ipsilateral axons away from the midline. In addition to this early function in guiding commissural axons towards the midline, MG are also fundamental later on to separate the commissures by enwrapping and subdividing them. Here, we show that the PDZ protein Cno and the DE-cadherin Shg participate in, and contribute to, the regulation of these later stage neural differentiation events, in which neuron-glial interactions play a central role.

**Cno forms a complex with Shg, Wrapper and Nrx-IV at the MG**

In *Drosophila*, Wrapper and Nrx-IV physically interact to promote glia-neuron intercellular adhesion at the MG (Stork et al., 2009; Wheeler et al., 2009). We propose that Cno and Shg are important components of this adhesion complex and key to its function. We found that both Cno and Shg are present at the MG, being highly restricted to the interface between MG and commissural axons. Cno and Shg were detected in a complex in vivo with Wrapper at the CNS MG. Nrx-IV, which is located on the surface of commissural axons, was also consistently found in a complex with Cno, although the amount of Cno protein that we were able to co-immunoprecipitate was much lower than that present in Cno-Wrapper complexes. One plausible explanation is that whereas Cno and Wrapper are present in the same cell (MG), Cno and Nrx-IV are in different cell types (MG and neurons, respectively) and, in addition, Cno is a cytoplasmic protein that is indirectly linked to Nrx-IV through other proteins in the same complex (i.e. Shg and Wrapper). Intriguingly, we found stronger genetic interactions between Cno and Nrx-IV than between Cno and Wrapper (double heterozygote analysis). A possible explanation for this is that Nrx-IV is not only acting through Wrapper-Shg-Cno in the MG but also through other partners, as previously proposed (Wheeler et al., 2009). In this way, when the dose of Cno and Wrapper was halved, Nrx-IV could still function fully through these other, putative partners.
Canoe function in neuron-glia interactions

Fig. 7. Shg localizes and functions at the MG in a complex with Wrapper. (A–C) Expression of Shg (green) at the MG. HRP is in magenta, Wrapper is in blue. Ventral view of three segments (A, A') showing Shg accumulation in a punctuate pattern around anterior and posterior commissures (arrows). In a sagittal view, Shg is also detected in close contact with commissures (B, B'). Shg colocalizes with Wrapper in the MG (C, C'). (D–G) Ventral views of stage 16 wild-type (D) and shg<sup>2</sup> mutant (E) Drosophila embryos stained for HRP (magenta) and Wrapper (green). Commissures are poorly separated in shg<sup>2</sup> mutants (arrowheads) and MG fail to enwrap the commissures (double arrows, compare with wild type). MG are also detected between commissures (single arrows). In sagittal views (F, G), defects in commissure ensheathment are apparent in shg<sup>2</sup> mutants (arrowheads), as well as the presence of MG between commissures (arrows in G, compare with F). (H–I) The subcellular localization of Cno is altered in shg<sup>2</sup> mutant embryos. Cno is in magenta, HRP in blue and Wrapper in green. Sagittal views of wild-type embryos show the localization of Cno within the MG at the contacts with the axonal commissures (arrows in H, H'). In shg<sup>2</sup> mutants, Cno is delocalized throughout the cytoplasm of MG (arrowheads in I'). (J) Co-IPs from wild-type embryo extracts show that Wrapper and Shg form a complex in vivo. (K) Model of neuron-glia interactions at the MG. The transmembrane protein Shg links the cytoplasmic protein Cno and the GPI-anchored protein Wrapper at the MG. Wrapper, in turn, binds to the transmembrane protein Nrx-IV present at commissural axons. The modular structure of all these proteins is shown (from NCBI ‘conserved domains’). RA, Ras-association domain; FHA, Forkhead; DIL, Dilute; PDZ (PSD-95, Discs large, ZO-1); GPI, glycosphingolipid; FN3, Fibronectin type 3; Ig-like, Immunoglobulin-like; TM, transmembrane; LamG, Laminin G; CA, Cadherin; FAS8C, Coagulation factor 5 or 8 C-terminal domain; EGF-like, Epidermal growth factor-like; CT, C-terminal. Scale bars: 10 μm.
However, halving the dose of Cno and Nr3-IV would impair not only the Nr3-IV-Wrapper-Cno signal but also the other potential pathways. In vertebrates, the ortholog of Nr3-IV, termed contactin-associated protein (Caspr or Cntnap) or paranoladin, is located at the septate-like junctions of the axonal paradomes, where it interacts in cis with contactin (at neurons) and in trans with neurofascin (at the glia) (Poliak and Peles, 2003). The Drosophila homologs of these Ig superfamily proteins, Contactin and Neurologin, interact in the same way with Nr3-IV at the septate junctions. However, there are no septate junctions at the neuron-MG interface (Jacobs and Goodman, 1989; Stollewerk et al., 1996) (see also below). Hence, other, as yet unknown partners of Nr3-IV might exist at this location.

Cno: more than an adherens junction protein
Cno and its vertebrate orthologs afadin/AF-6/Mllt4 have been shown to localize at epithelial adherens junctions (AJs), where they regulate the linkage of AJs to the actin cytoskeleton by binding both actin and Nectin family proteins (Lorger and Moelling, 2006; Mandai et al., 1997; Matsuo et al., 1999; Sawyer et al., 2009; Takahashi et al., 1998). However, Cno is not exclusively present at the AJs of epithelial tissues. Indeed, we previously found that Cno is also expressed in mesenchymal tissues, where it dynamically regulates three different signaling pathways required for muscle/heart progenitor specification (Carmina et al., 2006). The asymmetric division of these muscle/heart progenitors and of CNS progenitors also requires an AJ-independent function of Cno to asymmetrically locate cell fate determinants and properly orientate the mitotic spindle (Speicher et al., 2008). Therefore, Cno seems to act through different mechanisms depending on the cell type. Here, we describe a novel function of Cno during neurectodermal differentiation. In the MG, Cno, through Shg, contributes to the tight adhesion between the MG and the commissural axons and perhaps even to the regulation of some intracellular signaling within the MG. Indeed, Cno has been shown to regulate different signaling cascades during development (Carmina et al., 2006). To the best of our knowledge, no AJs or septate junctions (SJs) have been described at the MG-commissural axon interface (Jacobs and Goodman, 1989; Stollewerk et al., 1996). This suggests that the function of Cno in the midline is independent of AJs. In fact, the partner of Cno at this location, the Drosophila Nectin ortholog Echinoid (Wei et al., 2015), is not detected at the midline (our unpublished observations). In this context, it is worth pointing out that Shg is an epithelial cadherin key at AJs. Here, we have shown that Shg can also be found in non-epithelial tissues with an important function independent of AJs. A similar situation occurs with Nr3-IV. Despite Nr3-IV being a very well established component of SJs (Baumgartner et al., 1996; Faivre-Sarrailh et al., 2004; Schulte et al., 2003), no SJs are formed in the midline and no other known components of SJs are expressed there (Stork et al., 2009). Thus, different modes of Cno action, either as an AJ protein or as a signaling pathway regulator, are possible and they are not mutually exclusive: it all depends on the cell type and context.

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Competing interests statement
The authors declare no competing financial interests.

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