Development 140, 1507-1516 (2013) doi:10.1242/dev.092320

Src kinases mediate the interaction of the apical determinant Bazooka/PAR3 with STAT92E and increase signalling efficiency in *Drosophila* ectodermal cells

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SUMMARY

Intercellular communication depends on the correct organization of the signal transduction complexes. In many signalling pathways, the mechanisms controlling the overall cell polarity also localize components of these pathways to different domains of the plasma membrane. In the *Drosophila* ectoderm, the JAK/STAT pathway components are highly polarized with apical localization of the receptor, the associated kinase and the STAT92E protein itself. The apical localization of STAT92E is independent of the receptor complex and is due to its direct association with the apical determining protein Bazooka (Baz). Here, we find that Baz-STAT92E interaction depends on the presence of the *Drosophila* Src kinases. In the absence of Src, STAT92E cannot bind to Baz in cells or in whole embryos, and this correlates with an impairment of JAK/STAT signalling function. We believe that the requirement of Src proteins for STAT92E apical localization is mediated through Baz, as we can co-precipitate Src with Baz but not with STAT92E. This is the first time that a functional link between cell polarity, the JAK/STAT signalling pathway and the Src kinases has been established in a whole organism.

KEY WORDS: STAT92E, Bazooka/PAR3, Src kinases, Cell polarity, Signalling regulation

INTRODUCTION

Signalling pathways regulate development and homeostasis but their importance extends beyond normal physiological conditions, as mutations that ectopically activate or repress a signalling pathway are the cause of many human diseases. Protein tyrosine kinases are key elements of signal transduction in most pathways. In some cases, as in the EGF pathway, the transmembrane receptor itself has tyrosine kinase activity; in other cases, such as the JAK/STAT pathway, the intracellular domain of the receptor associates to a cytoplasmic tyrosine kinase.

The JAK/STAT pathway has been conserved during evolution, allowing *Drosophila melanogaster* to be used as a simplified model for signal transduction. In *Drosophila*, the pathway elements comprise only three ligands (Upd, Upd2 and Upd3), one homodimeric receptor (Dome), one JAK kinase (Hop) and one STAT transcription factor (STAT92E) (Arbouzova and Zeidler, 2006). This contrasts with the complexity found in mammals where multiple ligands and heterodimeric receptors, four JAK kinases and seven STAT proteins exist (Mohr et al., 2012). Despite millions of years of independent evolution, all studies show that the canonical signal transduction pathway is very similar in insects and mammals. In both systems, ligand binding to the receptor induces a change in conformation of the receptor complex that leads to the activation of its associated JAK kinases. These kinases transphosphorylate and phosphorylate the receptor on a tyrosine residue, creating docking sites for the inactive cytoplasmic STATs. STAT binding to the active receptor leads to its phosphorylation by JAK. Phospho-STAT proteins can homo- or heterodimerize through a conserved SH2 domain, allowing their stable translocation to the nucleus where they activate the transcription of target downstream genes (Mohr et al., 2012).

Despite this strong conservation, research in some model organisms has contributed information that still is not confirmed in others. For example, in mammals, where the pathway has been studied more extensively, it is known that unphosphorylated cytoplasmic STAT form dimers prior to their tyrosine phosphorylation. These STAT dimers have an antiparallel conformation different from the parallel conformation present in SH2 phospho-tyrosine dimers (Braunstein et al., 2003; Novak et al., 1998; Stancato et al., 1996). Moreover, in mammals, it has been shown that JAK is not the only kinase capable of STAT activation, but that Src tyrosine kinases can also activate STAT phosphorylation (Kazansky et al., 1999; Okutani et al., 2001; Silva, 2004). The Src family of non-receptor Tyr kinases comprises nine members in vertebrates, (reviewed by Thomas and Brugge, 1997), whereas in *Drosophila* only two members exist (Src42A and Src64B). The Src and STAT connection (Read et al., 2004; Silva and Shupnik, 2007), which has not been extensively studied in *Drosophila*, is especially interesting, as mutations in both proteins are linked to human cancer (Silva and Shupnik, 2007).

Studies in *Drosophila* have uncovered a strong relationship between ectoderm cell polarity and JAK/STAT signalling that have not yet been clearly established in vertebrate models (Sotillos et al., 2008). In *Drosophila* ectodermal tissues, the receptor, the JAK kinase and, surprisingly, the STAT transcription factor localize to the apical membrane. The apical polarization of the receptor and the JAK kinase affects the way the epithelium responds to the ligand, as cells can detect the ligands only when presented from the apical side but are unresponsive to ligands presented basally (Sotillos et al., 2008). In these ectodermal polarized tissues, the apical localization of STAT92E is functionally important, as it

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Accepted 16 January 2013

increases signalling efficiency, probably because the increased concentration on the apical side facilitates rapid translocation of the inactive cytoplasmic STAT92E to the activated receptor. It has been shown that the apical localization of STAT92E does not depend on DOME receptor binding or on pathway activation, but is due to STAT92E binding to the apical protein Bazooka (Baz, homolog of PAR3). Baz is a key component of the apical cell determining complexes in vertebrates and invertebrates, suggesting the possibility that JAK/STAT polarization may also occur in vertebrates (Hombría and Sotillos, 2008).

In this study, we have analysed the domains required for STAT92E and Baz interaction in *Drosophila*, and show that the Src tyrosine kinases are required for these two proteins to interact *in vivo*. This is the first time that a functional link between cell polarity complexes, the JAK/STAT signalling pathway and the Src kinases has been established in a whole organism. We also show data suggesting that, as in vertebrates, unphosphorylated cytoplasmic STAT92E could be forming dimers, extending the parallelism between the pathway in both lineages.

MATERIALS AND METHODS

Fly strains

We used the following alleles: *stat92E^{6346}*, *stat92E^{397}* (Brown et al., 2001; Hou et al., 1996; Silver and Montell, 2001); *Src42A$^{26.1}$* and *Src64B^{P1}* (Takahashi et al., 2005); Src64B^{KO} (O'Reilly et al., 2006) and STAT92E-GFP BAC [BAC CH321-73F24 P[acman] Genome Browser (Venken, 2009); a gift from Rebeca Spokony, modENCODE consortium]. This STAT92E-GFP BAC insertion is capable of rescuing the lethality and fertility of *stat92E^{397}/stat92E^{6346}* or *stat92E^{397}/stat92E^{83C9}* flies.

Null embryos for *STAT92E* were generated by inducing germ line clones in *hsFLP/+; FRT82B, stat92E^{6346}/FRT82B, ovo^{D1}* females heat shocked for 1 hour at 37°C at second instar larva, mated to UAS-X; *Klu-Gal4 stat92E^{6346}/TM6B* males (where *UAS-X* represents either *UAS-stat92E-GFP*; *UAS-stat92E-Cterm-GFP*; *UAS-stat92E-Nterm-GFP*; *UAS-stat92E^{Y711F}-GFP*; or a recombinant *UAS-stat92E-Myc, UAS-stat92E-Nterm-GFP*). Transgenic lines expressing *UAS-baz* (a gift from C. Doe, University of Oregon, USA), deletions of Baz (Krahn et al., 2010), *UAS-Src42A^{KM}* and *UAS-Src42A^{KY}* (Shindo et al., 2008), and *UAS-STAT92E-GFP* (Karsten et al., 2006) were used. We used *69B-Gal4*, *24B-Gal4* and *Klu-Gal4* as driver lines. The *crb43.2-lacZ* reporter gene has been described previously (Lovegrove et al., 2006) and *ems0.35-lacZ* is a fragment of *ems1.2-lacZ* (Jones and McGinnis, 1993).

Experiments in Fig. 5E were carried out by mating *Src42A$^{26.1}$*, *UAS-STAT92E-GFP/CyO wg-lacZ* females with *Src64B^{KO}*, *69B-Gal4/TM6B* males. Those embryos expressing STAT92E-GFP were heterozygous mutant for *Src42A$^{26.1}$* and *Src64B^{KO}*. For Fig. 5F, *Src42A$^{26.1}$*, *UAS-STAT92E-GFP/CyO wg-lacZ* females were mated to *Src42A$^{26.1}$/CyO wg-lacZ*; *Src64B^{KO}, 69B-Gal4/TM6B* males. As a result, embryos expressing STAT92E-GFP and not *lacZ* were homozygous mutant for *Src42A$^{26.1}$* and heterozygous for *Src64B^{KO}*.

For Fig. 5H,I, *Src42A$^{26.1}$*, *UAS-STAT92E-GFP/CyO wg-lacZ; Src64B^{KO}, UAS-baz/TM6B* females were mated to *Src42A$^{26.1}$/CyO wg-lacZ; 24B-Gal4/TM6B* males. Embryos expressing *lacZ* and expressing STAT92E-GFP and Baz in the mesoderm are heterozygous mutant for *Src42A$^{26.1}$* and *Src64B^{KO}* (Fig. 5H), whereas embryos expressing STAT92E-GFP and Baz in the mesoderm but not expressing *lacZ* are homozygous mutant embryos for *Src42A$^{26.1}$* and heterozygous for *Src64B^{KO}* (Fig. 5I).

For Fig. 5J,K, *Src42A$^{26.1}$, UAS-STAT92E-GFP/CyO wg-lacZ; UAS-baz*, *UAS-Src42A^{KM}/TM6B* (J) or *Src42A$^{26.1}$, UAS-STAT92E-GFP/CyO wg-lacZ; UAS-baz, UAS-Src42A^{YF}/TM6B* (K) females were mated to *Src42A$^{26.1}$/CyO wg-lacZ; Src64B^{KO}, 24B-Gal4/TM6B* males. Embryos not expressing *lacZ* and expressing *STAT92E-GFP* and *Baz* in the mesoderm, are homozygous for *Src42A$^{26.1}$* and heterozygous for *Src64B^{KO}*, and co-express the corresponding *UAS-Src42A* transgene.

For Fig. 6F,H *Src42A$^{26.1}$/CyO wg-lacZ; Src64B^{KO}/TM6B* females were mated to *Src42A$^{26.1}$/CyO wg-lacZ; crb-lacZ/ TM6B* males (Fig. 6F), or to *Src42A$^{26.1}$/CyO wg-lacZ; ems-lacZ/ TM6B* (Fig. 6H). Embryos homozygous for *Src42A$^{26.1}$* were identified by the absence of *wg-lacZ* staining pattern.

pUAST-STAT92E constructs

Fragments of STAT92E were amplified by PCR using High-Fidelity enzyme (Roche) using *pBs-STAT92E* as a template and the following primers: STAT92E Nterm 5′, ACGAATTCGAGCATGAGCTTGTGG; STAT92E Nterm 3′, ACGGATCCAGGTTCTTCTGCGCG; STAT92E Coiled-coil domain 5′, ACGAATTCATGCCGCTAAATCCC; STAT92E Coiled-coil domain 3′, ACGGATCCAGGTTCTTCTGCGCG; STAT92E DNA-binding domain 5′, CAGAATTCATGGCCTTTATCG; STAT92E DNA-binding domain 3′, ATGGGATCCATGTGATCTTTGG; STAT92E Cterm 5′, CAGAATTCATGGCCTTTATCG; STAT92E Cterm 3′, GTGA-ACAGCTCCTCGCCC; STAT92E CtermΔTAD 5′, CAGAATTCAT-GGCCTTTATCG; STAT92E CtermΔTAD 3′, CTGGATCCCTCTTC-ACGGGAC; STAT92E CtermΔ725-761 5′, CAGAATTCATGGCC-TTTATCG; STAT92E CtermΔ725-761 3′, TCGGATCCTCTCCGTTTCTAC

After sequencing, the cDNAs were subcloned into the *Eco*RI-*Bam*HI sites of *pUAST-GFP*. To generate the *UAS-STAT92E-Myc* the STAT92E cDNA was cloned in *Eco*RI in pUAST-Myc (Sotillos et al., 2008).

Immunohistochemistry and cuticle preparations

Embryos were fixed in 1:1 formaldehyde 4% in PBS:n-heptane for 20 minutes at room temperature and stained with the following primary antibodies: anti-Crb, anti-α-Spectrin, anti-Neurotactin (Developmental Studies Hybridoma Bank), anti-Myc (Cell Signaling), anti-Scrib (a gift from C. Doe, University of Oregon, USA), anti-Baz (a gift from A. Wodarz, GZMB, Göttingen, Germany), anti-Src42A (a gift from M. Takahashi, University of Tokyo, Japan) anti-aPKC C20 (Santa Cruz Biotechnology) and anti-βGAL (Promega). Secondary antibodies were coupled to Alexa488, Alexa555 or Alexa648 (Molecular Probes).

Cells were fixed in 4% formaldehyde, phosphate buffer (pH 7.4). Primary antibodies were mouse anti-Myc (9E10, Developmental Studies Hybridoma Bank, 1:100) and rat anti-HA (Roche #11867423001, 1:1000).

DNA was stained with DAPI (Molecular Probes). We used Cy3-conjugated (Jackson Laboratories) or Alexa647-conjugated (Invitrogen) secondary antibodies. Images were taken on a Zeiss LSM 510 Meta confocal microscope and processed using Adobe Photoshop.

Cuticle preparations were carried out as described previously (Hombría et al., 2005). The frequency of segmentation and spiracle defects observed in the late embryo cuticles presented in Fig. 6 are as follows: *stat92E^{6346}/+* females mated to Oregon R wild-type males (0%, *n*=86). *Src42A$^{26.1}$/+* females mated to *Src64B^{P1}/+* males (0%, *n*=145). *Src42A$^{26.1}$/+; stat^{6346}/+* females mated to Oregon R males (30%, *n*=92). *Src42A$^{26.1}$/+; stat^{6346}/+* females mated to *Src64B^{P1}/+* males (30%, *n*=76).

Immunoprecipitation, pull-down assays and western blots

For immunoprecipitation, 1 μg of mouse anti-GFP (Roche), mouse anti-βGAL (Promega), rabbit anti-βGAL (Cappel) or 5 μl of rabbit anti-Baz were added to *UAS-STAT92EGFP/+; 69B-Gal4/UAS-baz* embryonic extracts containing 3 mg of total protein. TNT buffer [1% Triton X-100, 150 mM NaCl, Tris-Cl (pH 7.5)] was used for immunoprecipitation with anti-Baz, and STAT buffer (0.1% Triton X-100, 300 mM NaCl, 50 mM Tris-Cl pH 7.5, 5 mM EDTA) for immunoprecipitation with anti-GFP. Buffers were supplemented with protease inhibitors (Roche), β-glycerophosphate and 20 mg/ml sodium orthovanadate. Antibody conjugation with protein A/G Plus-Agarose (Santa Cruz) was carried out overnight at 4°C. Embryos were lysed in the proper buffer and centrifuged for 30 minutes at maximum speed at 4°C to discard debris. Total protein (2 mg) was used in each assay and preabsorbed for 30 minutes with protein A-Agarose (Santa Cruz). Before incubation with the antibody, 5 μl of each assay was removed as loading control (input). Immunoprecipitations were performed at 4°C for 4 hours. Immune complexes were washed four times in washing buffer [300 mM NaCl, 50 mM Tris-Cl (pH 7.5), 5 mM EDTA and 0.1% Triton X-100] and once more in 50 mM Tris-Cl (pH 6.8) or PBS, boiled in 2×SDS sample buffer before SDS-PAGE and western blot.

To perform pull-down experiments, GST fusion proteins were obtained from subclones in pGEX4T1 (Amersham) of Baz$^{1-317}$ or Baz$^{1048-1464}$ (Krahn et al., 2010). Purification was carried out using standard methods.

Drosophila protein extract (300 µg) in TNT buffer or 30 µl of ^{35}S -labelled STAT92E protein in STAT buffer were incubated for 5 hours at 4°C with 30 µg of Glutathione-S-transferase (GST) or with the different GST-fusion proteins bound to glutathione beads as described previously (Sotillos et al., 2004). Complexes were washed as immunoprecipitations. Western blotting was carried out according to standard procedures using rabbit anti-Baz and mouse anti-GFP (Roche) at 1:2000. When using ^{35}S-labelled STAT92E after electrophoresis, the gel was dried and detected by phosphorimager method.

Schneider 2R+ cells were transfected with the following constructs: Poly-Ubiquitin::Baz-GFP, Poly-Ubiquitin::Src42A-HA and Poly-Ubiquitin::STAT92E-myc. Cells were harvested 3 days after transfection and lysed in lysis buffer [1% Triton X-100, 150 mM NaCl, 50 mM Tris-Cl (pH 7.5)] supplemented with protease inhibitors. After centrifugation, 2 µl of rabbit anti-GFP (Molecular Probes #A11122) were added to the cell lysate. Immune complexes were harvested using protein A-conjugated agarose (Roche), washed five times in lysis buffer and boiled in 2×SDS sample buffer before SDS-PAGE and western blot. Western blot was performed using mouse anti-Myc (9E10, Developmental Studies Hybridoma Bank, 1:200), mouse anti-HA (Roche #11 583 816 001, 1:1000) or rabbit anti-GFP (Molecular Probes #A11122, 1:1000).

RESULTS

All vertebrate STAT proteins share a conserved domain structure whose function has been well studied in mammals. The domains include: a non-conserved N-terminal region that varies in different STATs, a coiled-coil, a DNA-binding, a linker, an SH2 and a C terminus *trans* activation domain (Fig. 1A) (Becker et al., 1998; Chen et al., 2002; Vinkemeier et al., 1998). The N-terminal domain is required for the formation of tetramers, for tyrosine dephosphorylation, for transcriptional activation and for protein-protein interactions (Chang et al., 2003; Chen et al., 2002; Murphy et al., 2000; Ota et al., 2004; Shuai et al., 1996; Vinkemeier et al., 1998; Xu et al., 1996). The helical coiled-coil domain mediates interactions with several proteins, including Jun (Zhang et al., 1999) and is also required for the formation of non-phosphorylated dimers (Mao et al., 2005; Neculai et al., 2005). The DNA-binding domain (DB) has limited contact with both the major and minor grooves of DNA (Chen et al., 1998). The linker domain modulates the rate of STAT:DNA interactions, ultimately controlling transcriptional activation of STAT target genes (Yang et al., 2002). The SH2 domain is required for the formation of an activated STAT dimer by mediating reciprocal interactions with a phosphorylated conserved tyrosine residue located around amino acid position 700 that exists in all STAT proteins (Chen et al., 1998; Levy and Darnell, 2002). Last, the C-terminal transactivation domain (TA) is required for transcriptional co-activation of mammalian STATs (Horvath, 2000).

Domains required for STAT92E apical localization

The *Drosophila* STAT92E protein can be similarly subdivided (Fig. 1A) with the N-terminal domain spanning amino acid residues 1-139, the helical coiled-coil domain spanning residues 140-340, the DNA-binding domain spanning residues 340 to 588, the SH2 domain spanning residues 594-659 and the terminal TA domain spanning residues 659-761 (Hou et al., 1996; Yan et al., 1996). As the requirement of these domains has not been defined in relation to the apical localization of STAT92E, we analysed this specific aspect in the different fragments.

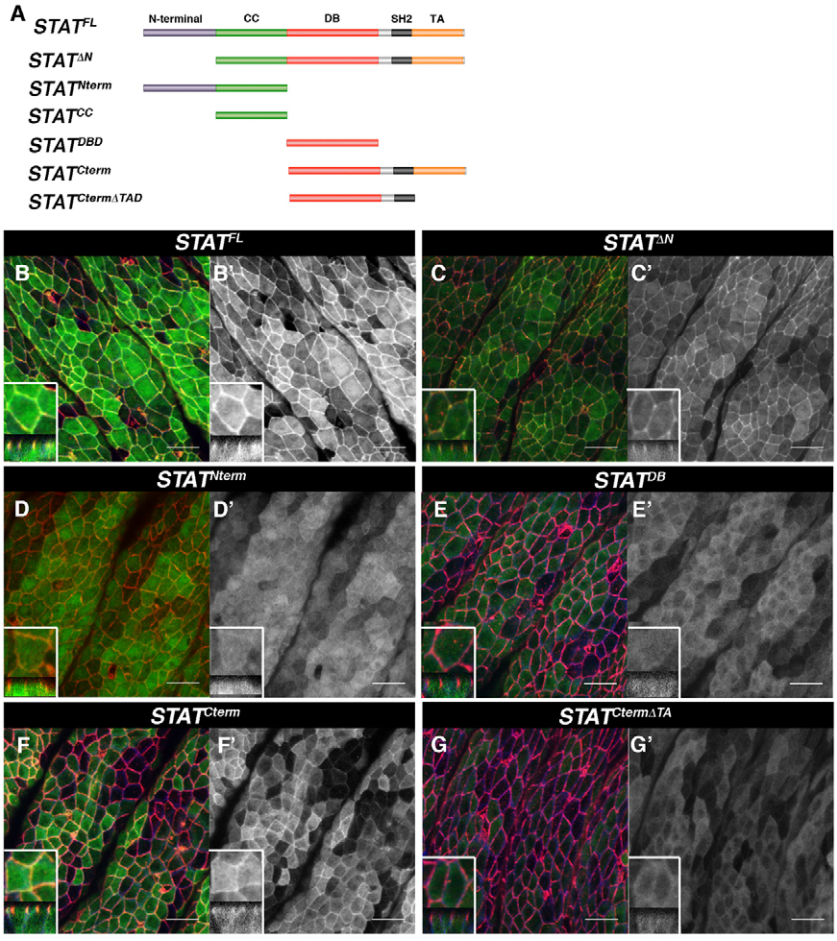

Fig. 1. Domains required for STAT92E subapical membrane localization. (**A**) Schematic representation of full-length protein and protein variants tested. Purple, N-terminal domain; green, coiled-coil domain; red, DNA-binding domain; black, SH2 domain; orange, transactivation domain. All fragments were C-terminally tagged with GFP and expressed in the epidermis of embryos using the GAL4 system. (B-G) Panels show the ectoderm of stage 15 embryos expressing the indicated constructs under the control of *69B-Gal4* stained with the subapical marker anti-Baz (red), the basolateral marker anti-Dlg (blue, B,C,E,G) and GFP (green, or grey in B′-G′). (**B**,**B′**) Full-length STAT92E localizes to the cytoplasm and concentrates in the subapical membrane. (**C**,**C′**) The N-terminal domain is dispensable for STAT92E subapical membrane localization. (**D**,**D′**) The N-terminal half of the protein, including the N-terminal and the coiled-coil domains are detected subapically, albeit at much lower levels than the full-length protein (the yellow at the membrane in D is due to the weak colocalization). (**E**,**E′**) The isolated DNA-binding domain is cytoplasmic. (**F-G′**) The C-terminal half of the protein (F,F′) localizes to the subapical membrane as efficiently as the full-length STAT92E, but the C-terminal domain lacking the transactivation domain (G,G′) remains in the cytoplasm. Insets show higher magnifications and cross-sections. Scale bars: 10 µm.

Using the Gal4/UAS (Brand and Perrimon, 1993) system, we expressed in the embryonic ectoderm different STAT92E fragments fused to GFP and analysed their membrane localization (Fig. 1; for comparison of expression levels, see also supplementary material Fig. S1). The full-length protein localizes to the subapical membrane of the cells (Fig. 1B,B′) as was previously described for the endogenous protein (Sotillos et al., 2008). Similar results were obtained with a STAT92E-GFP fusion under the control of the genomic region (STAT92E-GFP BAC, supplementary material Fig. S2) capable of rescuing STAT92E mutants to adulthood Deletion of the N-terminal non-conserved domain does not affect membrane localization (STAT92E-ΔN, Fig. 1C,C′). Constructs containing the coiled-coil domain plus the N-terminal domain (STAT92E-Nterm, Fig. 1D,D′), or the coiled-coil domain on its own (STAT92E-CC, not shown) localize to the membrane, although at much lower levels than the full-length STAT92E. By contrast, the DNA-binding domain shows no membrane localization (STAT92E-DB, Fig. 1E,E′). The C-terminal half of STAT92E, including the DNA-binding, the SH2 and the TA domains, also localizes to the membrane in ectodermal cells (STAT92E-Cterm, Fig. 1F,F′). These results indicate that both the N-and the C-terminal part of the protein have the potential to translocate STAT92E to the apical cell membrane.

In vertebrates, it has been described that non-phosphorylated STATs can form antiparallel dimers (Braunstein et al., 2003; Novak et al., 1998; Stancato et al., 1996). To test whether dimerization with the endogenous STAT92E could be the cause of the membrane localization of some of our constructs, we studied their distribution in embryos completely lacking the endogenous STAT92E protein. For that purpose, we studied the progeny of STAT92E mutants originating from STAT92E germ line clones to get rid of both the maternal and zygotic STAT92E protein. In these STAT92E-null embryos, tagged full-length STAT92E (Fig. 2A) and the STAT92E C-terminal half (Fig. 2C) can still localize to the membrane. By contrast, the N-terminal half loses its faint apical localization in STAT92E-null embryos (Fig. 2B), suggesting that its apical membrane localization in wild-type embryos is due to interaction with the endogenous STAT92E protein. Moreover, in STAT92E-null embryos STAT92E N-terminal localization to the membrane is restored when co-expressed with a full-length Myc tagged STAT92E (Fig. 2D). As the N-terminal half does not contain the SH2 domain, these results indicate that the *Drosophila* cytoplasmic STAT92E protein dimerizes prior to tyrosine 711 phosphorylation. However, although we cannot ignore the possibility that another protein is acting as a bridge between both STAT92E fragments, considering that vertebrate unphosphorylated STAT proteins dimerize, dimerization with the endogenous apically localized STAT92E is most probably responsible for the weak polarization observed in the N-terminal fragments (Fig. 1D).

These data also suggest that the C-terminal half of STAT92E binds to apically localized factors. As our previous work demonstrated that Baz is required for STAT92E membrane localization (Sotillos et al., 2008), we studied the ability of different STAT92E fragments to co-immunoprecipitate with Baz. Only STAT92E full-length and the STAT92E C-terminal half co-precipitate with Baz from embryonic extracts (Fig. 3A,B).

The C-terminal half of STAT92E contains the DB domain, the linker region, the SH2 domain and the TA domain. When the TA domain is removed from the STAT92E C-terminal half, the resulting fragment loses the membrane localization (Fig. 1G,G′ and supplementary material Fig. S3) and most of its capability of Baz binding (Fig. 3C). These results indicate that the TA domain is required for the interaction with Baz and the apical membrane localization of STAT92E.

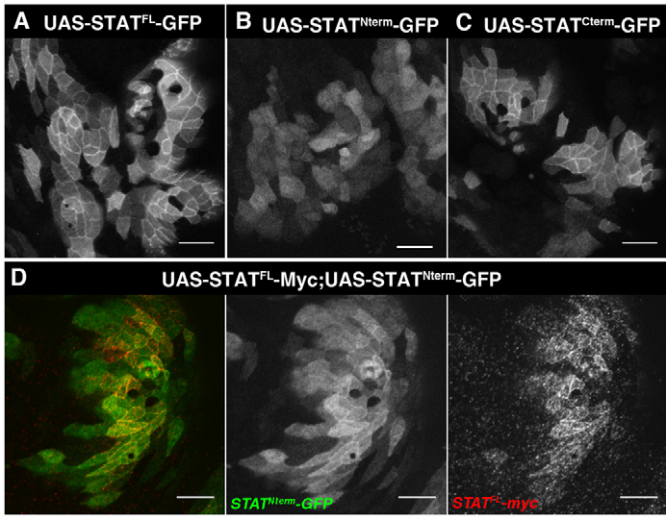

Fig. 2. STAT92E homodimerization and membrane localization domains. (**A-C**) Ectodermal expression of full-length STAT92E (A), or the N-terminal (B) or the C-terminal (C) half of the protein in germline clone embryos lacking the endogenous STAT92E. In *stat92E*-null embryos the full-length (A) and C-terminal half (C) localize to the membrane, whereas the N-terminal half (B) is unable to localize to the apical membrane. (**D**) In this same background, the N-terminal half regains weak apical membrane localization when co-expressed with full-length STAT92E-Myc. (A-C) GFP staining. (D) GFP on the middle and anti-Myc on the right panel with merged channels on the left. Scale bars: 10 μm. All pictures are centred in posterior spiracle region. Anterior is leftwards and dorsal is upwards.

Baz protein domains required for STAT92E localization to the apical membrane

Baz is a conserved scaffold protein involved in the establishment of apico-basal polarity in many organisms (Suzuki and Ohno, 2006). Baz contains three conserved regions (CR) (Fig. 4B) with established functions (Benton and St Johnston, 2003a; Izumi et al., 1998; Joberty et al., 2000; Lin et al., 2000; Morais-de-Sá et al., 2010; von Stein et al., 2005). The N-terminal CR1 is required for homodimerization; the CR2 includes three PDZ domains and is involved in the interaction with PAR6; the C-terminal CR3 interacts with aPKC. Apart from these, Baz contains two phosphorylation sites for PAR1, which serve as binding sites for 14-3-3 adaptor proteins, and the C-terminal region interacts with membrane lipids and mediates direct interaction with the plasma membrane (Benton and St Johnston, 2003b; Krahn et al., 2010). In the wild-type embryos, Baz is not expressed in the mesoderm and STAT92E does not localize to the membrane cortex in this tissue. However, we have shown (Sotillos et al., 2008) that STAT92E is translocated towards the membrane upon ectopic expression of Baz in the mesoderm (compare Fig. 4A,C and supplementary material Fig. S3A,B). To find out which region of Baz is required for STAT92E translocation to the cortex, we expressed different fragments of the Baz protein fused to GFP in mesodermal cells and observed which fragments could affect the localization of a full-length *STAT92E*-Myc-tagged protein. We find that the PDZ and the aPKC-binding domains are dispensable for STAT92E membrane translocation (Fig. 4E,F). However, when we remove either the most N-terminal (amino acids 1-317) or C-terminal regions (a complete deletion of the C-terminal region, amino acids 1001-1464; or a smaller deletion, amino acids

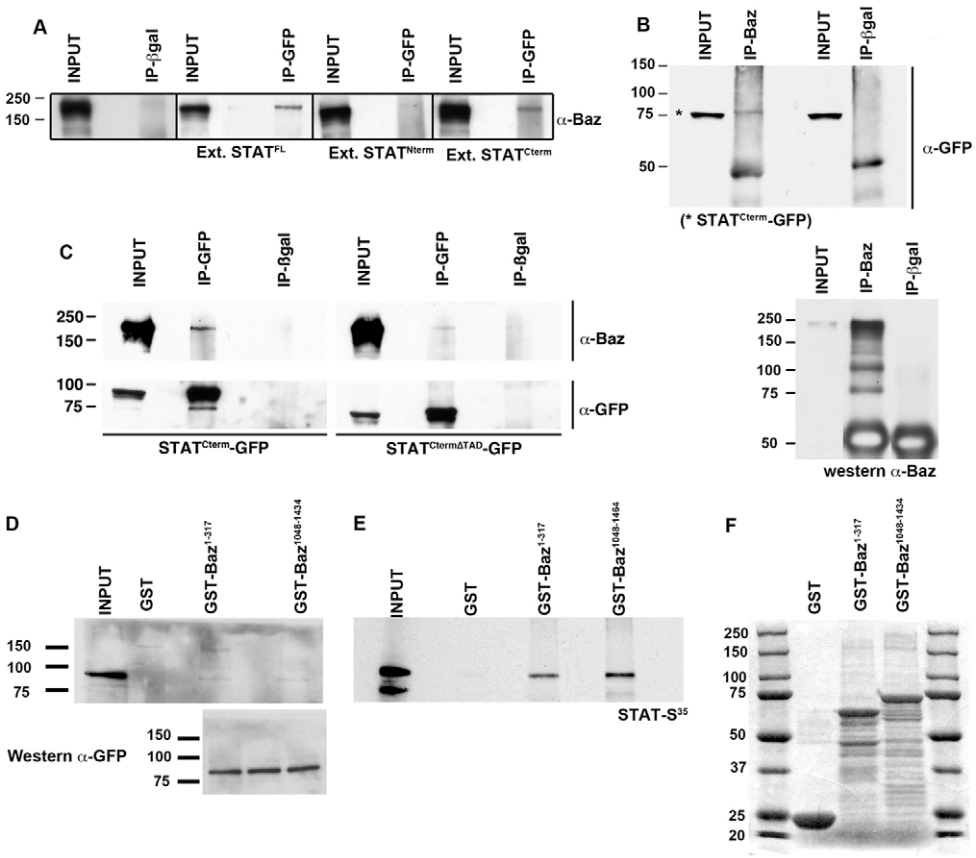

Fig. 3. Baz and STAT92E domains required for interaction. (**A**) The C-terminal region of STAT92E co-precipitates with Baz from embryonic extracts, as does the full-length protein, whereas the N-terminal region does not co-precipitate. (**B**) Baz is able to immunoprecipitate with the C-terminal region of STAT92E from embryonic extracts. Anti-β-gal antibody was used as a control. Control of Baz immunoprecipitation is shown below. (**C**) Baz is not able to immunoprecipitate from embryonic extracts with a C-terminal region of STAT92E in which the TA domain has been removed. (**D**,**E**) GST-Baz fragments or control GST were incubated with embryonic extracts expressing STAT92E-GFP (D) or STAT92E transcribed *in vitro* in the presence of Met-S35 (E). Both the N- and the C-terminal regions of Baz co-immunoprecipitate with STAT92E. Equal amounts of embryonic extracts were used in the three pull-downs (bottom panel in D). (**F**) Coomassie-stained SDS-PAGE of purified GST fusion proteins used for the pull-down assays. Molecular weight markers are indicated on each panel.

1221-1464) of the protein, Baz cannot translocate STAT92E towards the membrane (Fig. 4D,G,H). To confirm these results, we performed pull-down assays and found that both the N- (1-317) and the C-terminal (1048-1464) regions of Baz can co-precipitate with STAT92E from embryonic extracts (Fig. 3D). We obtained the same results with *in vitro* pull-down assays expressing STAT92E in rabbit reticulocyte extracts (Fig. 3E). Thus, our results point to a function of both, the N- and the C-terminal region of Baz in STAT92E membrane localization.

The STAT92E-Baz interaction requires Src activity

To test whether Baz is sufficient for STAT92E membrane localization, we co-expressed both proteins in S2R+ cell lines. Surprisingly, in these *ex vivo* conditions we were unable to see STAT92E membrane translocation (Fig. 5A; 82% of cells show cytoplasmic STAT92E and only 18% of cells show cortical STAT92E, n=50). As we had observed that Baz and STAT92E *in vivo* co-precipitation is detected only in the presence of phosphatase inhibitors (this work) and we had previously shown that JAK phosphorylation is dispensable for STAT92E-Baz interaction [STAT92E still localizes to the membrane in null mutants for JAK or when the Tyr phosphorylated by JAK is mutated (Sotillos et al., 2008)], we suspected that other kinases might be involved in this process. Because members of the Src kinase family have been previously related with JAK/STAT signalling in vertebrates and invertebrates (Read et al., 2004; Silva and Shupnik, 2007), we investigated their possible involvement in this process. We observe that expression of Src42A or Src64B did not affect STAT cytoplasmic localization (Fig. 5B, 72% of cells show cytoplasmic STAT92E). However, when we express either Src42A or Src64B in the presence of Baz, this leads to STAT92E cortical translocation in S2R+ cells (Fig. 5C, 86% of cells show STAT92E cortical colocalization and only in 14% STAT92E remains in the cytoplasm). Moreover, we were able to co-immunoprecipitate Src42A with Baz from transfected S2R+ cells (Fig. 5D).

In wild-type ectodermal cells, STAT92E and Src42A colocalize at the apical junctional region. Moreover, in mesodermal cells, Src42A also colocalizes with STAT92E after Baz overexpression (supplementary material Fig. S5). To confirm that Src is required for STAT92E cortex localization, we studied the epidermal membrane distribution of STAT92E in *Src* mutants, and the ability of Baz to translocate STAT92E to the membrane in mesodermal cells with depleted *Src* levels. In *Src42A/+; Src64B/+* double heterozygous ectodermal cells, STAT92E-GFP membrane localization is not noticeably affected (Fig. 5E). However, in *Src42A/Src42A; Src64B/+* embryos, there is a reduction of STAT92E at the membrane without any effect on Baz localization (compare Fig. 5E,F, and supplementary material Fig. S6). The requirement of Src for STAT92E cortical membrane localization is observed more clearly in the pharynx, where there is a gradual reduction of STAT92E membrane localization that correlates with the number of functional *Src* alleles in the embryo: high levels when Baz is expressed in the wild type; intermediate levels when expressed in double heterozygous *Src42A/+*; *Src64B/+* embryos; and residual STAT92E localization when expressed in *Src42A/Src42A; Src64B/+* embryos (compare Fig. 5G with 5H,I, and supplementary material Fig. S6). These experiments show that Src is required for Baz-mediated recruitment of STAT92E.

To test whether the abnormal STAT92E membrane localization in *Src* mutants correlates with a decrease of JAK/STAT signalling *in vivo*, we examined whether *Src* mutations increase the phenotype of a *stat92E* partial loss of function. During embryogenesis, STAT92E

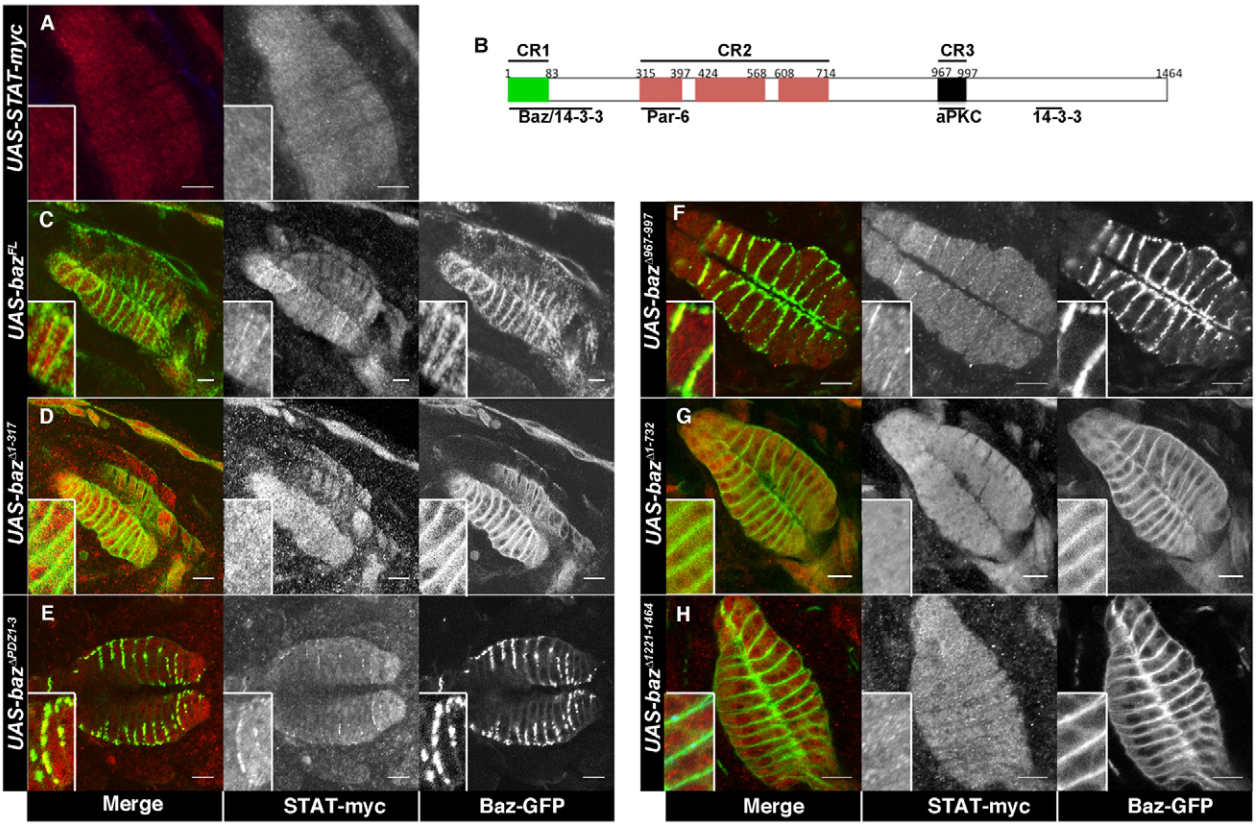

Fig. 4. Baz domains required for STAT92E membrane localization. (**A**) STAT92E-Myc expressed in the pharynx of wild-type embryos does not localize to the membrane. (**B**) Schematic representation of Baz. (**C-H**) Co-expression of STAT92E-Myc with: full-length GFP-Baz (C), GFP-BazΔ1-317 (D), GFP-BazΔPDZ1-3 (E), GFP-BazΔ967-997 (F), GFP-BazΔ1-732 (G) and GFP-BazΔ1221-1464 (H). Full-length Baz can relocate STAT92E to the membrane of mesodermal cells (C), but translocation does not occur when the N-terminal region (D,G) or the C-terminal region (H) of Baz are removed, whereas the PDZ- and the aPKC-binding domains are dispensable for STAT92E translocation (E,F). STAT92E-Myc (red; grey in the centre panels) GFP-Baz (green; grey in the right-hand panels). Insets show higher magnifications. Scale bars: 10 μm. Anterior is leftwards.

is required for head involution, segmentation and posterior spiracle formation. Heterozygous *stat92E/+* mutant embryos do not present any defects (Fig. 6A) but provide a sensitized background to screen for mutations affecting JAK/STAT signalling efficiency (Sotillos et al., 2008). Similarly, heterozygous embryos for *Src42A/+* or *Src64B/+*, or double heterozygous *Src42A/+; Src64B/+* have normal cuticles (not shown and Fig. 6B). However, *Src42A/+; stat92E/+* heterozygous embryos present defects in head formation, segmentation and posterior spiracle development (Fig. 6C), and these phenotypes are enhanced in *Src42A/+; Src64B/+; stat92E/+* embryos (Fig. 6D). We also studied how the levels of Src affected the expression of the spiracle *crumbs* enhancer *crb843.2*, a direct target of the JAK/STAT pathway (Fig. 6E) (Lovegrove et al., 2006). The levels of activation of *crb843.2* are severely reduced in *Src42A/Src42A; Src64B/+* embryos (Fig. 6F), whereas the expression of *ems0.35*, another spiracle enhancer activated independently of the JAK/STAT pathway is unaffected (compare Fig. 6I with 6H). In parallel to the observed reduction of JAK/STAT signalling, these *Src* mutant embryos show altered spiracle morphology (Fig. 6G).

Data from vertebrates have shown that Src kinases are able to activate STAT independently of JAK function by direct phosphorylation of the same conserved Tyr involved in STAT dimerization (Okutani et al., 2001; Silva, 2004). We excluded the possibility that Src phosphorylation of this tyrosine is responsible for STAT92E membrane translocation, as we can detect the mutated STAT92E-Y711F at the membrane cortex of ectodermal cells in STAT92E-null embryos (Fig. 5L). This agrees with our previous observation that STAT92E membrane localization also occurs in cells where the pathway is not active (Sotillos et al., 2008). To test the requirement of the kinase activity of Src to mediate STAT92E-Baz interaction, we studied the rescue capability of a Src kinase dead ($Src42A^{KM}$) versus a constitutively activated Src42A ($Src42A^{KY}$) in mesodermal cells. Both forms were able to rescue to the same extent (Fig. 5J,K and supplementary material Fig. S6), indicating that the kinase activity of Src42A is not required for this interaction.

Thus, our data uncover a novel function for the Src kinases in JAK/STAT signalling where Src regulates a Baz-STAT92E membrane interaction required for efficient signal transduction.

DISCUSSION

Maintenance of cell polarity is crucial for the correct cellular function. Not only most of the cellular processes are compartmentalized but the cell membrane polarity also affects signal transduction in several pathways. Here we uncover the relationship between Baz, a key molecule involved in the establishment and maintenance of cell polarity; STAT92E, a transcription factor of a major signalling pathway; and the Src kinases, which play a role in signal transduction and cell polarity. All three proteins have been associated with different kinds of tumours (Huang and Muthuswamy, 2010; Silva and Shupnik, 2007)

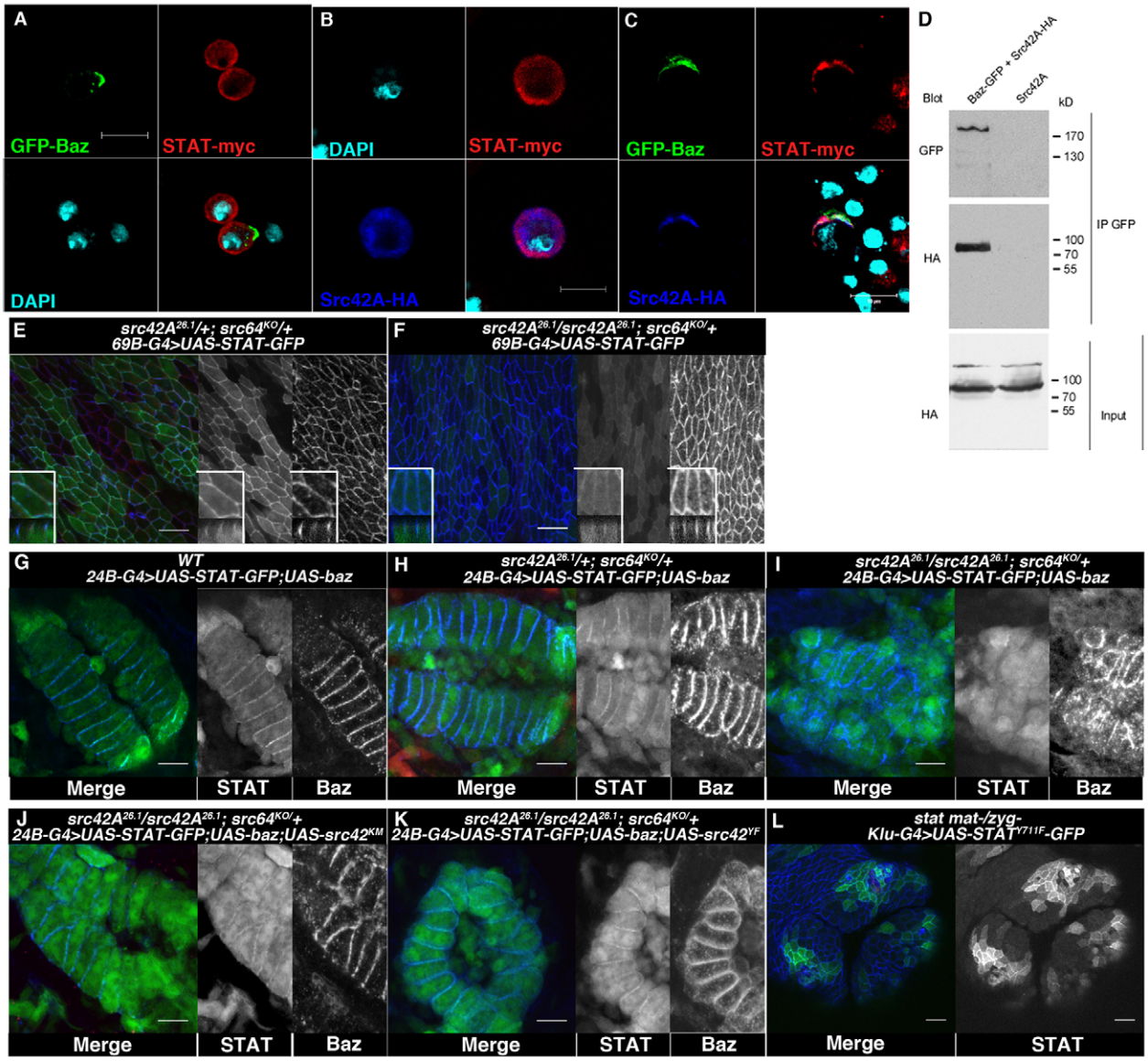

Fig. 5. Src requirement for STAT92E and Baz colocalization. (**A-C**) Subcellular localization of STAT92E-Myc fusion proteins in S2R+ cells. Cells were co-transfected with *UAS-STAT92E-Myc*, *UAS-GFP-Baz* constructs and *actin5C-GAL4*, and stained for Myc (red), GFP (green) and DAPI (blue) in the absence (A) or the presence (C) of *UAS-Src42A-HA* (dark blue). Control cells co-transfected with *UAS-STAT92E-Myc* (red) and *UAS-Src42A-HA* (dark blue) are shown in B. (**D**) Co-immunoprecipitation after transfection of GFP-Baz and Src42A-HA in S2R+ cells. S2R+ Src42A-HA lysate input down. (**E**,**F**) Requirement of Src for STAT92E membrane localization. STAT92E membrane localization (central panels) is strongly reduced when the two copies of *Src42A* and one copy of *Src64B* are removed (F) compared with a *Src42A* heterozygous background (E). (**G-I**) Baz recruitment of STAT92E to the membrane of pharynx cells is impaired in *Src* mutant backgrounds. In mesodermal cells, STAT92E localizes to the membrane if Baz is co-expressed (G). This recruitment is compromised in double heterozygous *Src42A/+; Src64B/+* mutant embryos (H), and nearly abolished when the two copies of *Src42A* and one of *Src64B* are removed (I). (**J**,**K**) In the same mutant background as I, embryos co-expressing either *UAS-Src42A*^{KM} (J) or *UAS-Src42A*^{KY} (K) show STAT92E in the membrane of pharyngeal cells. (**L**) STAT92E Tyr711 phosphorylation is not required for membrane localization. Expression of *STAT92E* with the conserved tyrosine 711 mutated to phenylalanine is able to localize in the membrane of null *stat92E* embryos (GFP in grey on the right panel). (E-K) GFP is shown in the middle panels and anti-Baz in the right panels. Insets show higher magnifications and cross-sections. Scale bars: 10 μm. Anterior is leftwards.

including epithelial tissue carcinomas and breast cancer. However, this is the first time that these three proteins have been found to function together in a cellular process in the whole organism and a common mechanism of action is proposed.

Src kinases regulate STAT92E membrane localization

In vertebrates, there is strong evidence showing that, besides JAK, the Src kinases can activate STAT signalling. First, STAT3 and STAT5 are crucial downstream factors in Src-induced transformation (Bromberg et al., 1998; Kloth et al., 2003). Second, Src kinase activation has been shown to result in STAT tyrosine phosphorylation (Okutani et al., 2001; Silva, 2004). By contrast, in *Drosophila* the available data suggest that the involvement of Src in STAT92E activation is probably marginal. This is shown by the fact that in *Drosophila* the phenotypes of null *stat92E* alleles are very similar to those of mutants in which the function of the JAK kinase, the receptor or of all ligands, is abolished, indicating that STAT92E

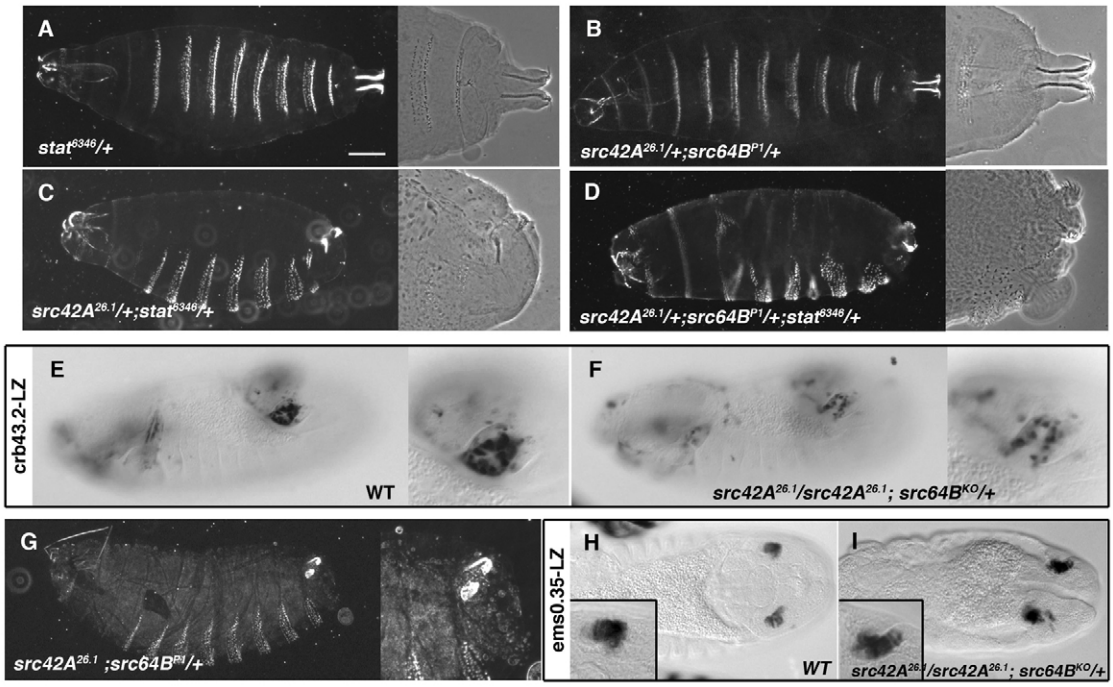

Fig. 6. Src requirement for STAT92E function. (**A-D**) Genetic interactions between *STAT92E*, *Src42A* and *Src64B*. (A) Heterozygous *stat92E/+* embryos are viable and show no defects. (B) *Src42A/+; Src64B/+* double heterozygous embryos are also normal (0% defects, *n*=106). (C) *Src42A/+; stat92E/+* double heterozygous embryos display segmentation and spiracle defects resembling JAK/STAT pathway signalling hypomorphic phenotypes (30%, *n*=94). The phenotype is enhanced when a single *Src64B* copy is also removed (D, 30%, *n*=65). (**E-G**) Expression of *crb43.2-lacZ* (E) is strongly reduced in 45% of stage 12 *Src42A/Src42A; Src64B/+* embryos (F, *n*=38). Accordingly, these embryos show posterior spiracle defects (G, 20%, *n*=124). (**H**,**I**) The spiracle reporter gene *ems0.35* (H) activated independently of JAK/STAT signalling is not affected in a Src mutant background (I). Higher magnifications of spiracles are shown on the right (A-G) or in the bottom left-hand corners (H,I) of each panel. Scale bars: 10 μm. Anterior is leftwards.

activation via alternative kinases occurs in a minority of tissues. One of the few cases reported of JAK-independent STAT92E activation occurs during the proliferation and migration of the embryo pole cells where STAT92E is activated by the Ras/Raf pathway downstream of the Torso tyrosine kinase receptor (Li et al., 2003). The study of mutations in the C-terminal Src kinase (Csk), a negative regulator of Src signalling, offers indirect evidence to suggest that Src may activate STAT92E in the *Drosophila* eye. Eyes that lack Csk are larger than normal, a phenotype also observed when the canonical JAK/STAT pathway is ectopically activated during eye development. Moreover, Csk clones exhibit higher levels of STAT92E expression, which has been interpreted as being due to the induction of a STAT92E positive-feedback loop (Read et al., 2004). However, these results do not clarify whether Src-induced STAT92E activation is due to direct STAT92E phosphorylation or caused by indirect regulation.

In the ectoderm, where the JAK/STAT pathway is highly polarized, we have shown that efficient signalling requires STAT92E apical localization achieved through interaction with the membrane-associated apical polarity protein Baz (Sotillos et al., 2008). Here, we have obtained several pieces of evidence showing that Src is also required for the correct STAT92E membrane localization and signalling (Figs 5, 6). First, although heterozygous *stat92E* or *Src* mutant embryos are normal, double heterozygous *stat92E* and *Src* mutant embryos display phenotypes that resemble a partial JAK/STAT signalling failure. Second, the expression of a direct target of STAT92E is downregulated in a *Src* mutant background. Third, decrease of *Src* gene activity affects STAT92E localization to the membrane of epithelial cells or to the membrane of mesodermal cells expressing Baz. Fourth, in S2R+ cells STAT92E co-localizes with Baz only when co-expressed with Src42A or Src64B. Our data also show that tyrosine 711 is not required for membrane localization, demonstrating that this function of Src is independent of STAT92E activation.

In our experiments, although we are able to co-precipitate Baz with STAT92E and Baz with Src42A, we could not co-precipitate Src42A with STAT92E. This suggests that the interaction between Src42A and STAT92E is either too labile to be detected or that Src modifies Baz and that this allows the recruitment of STAT92E to Baz. Our data also suggest that the kinase activity of Src42A is dispensable for STAT92E-Baz interactions, as a kinase-dead isoform of Src42A is able to rescue membrane localization in *Src* mutant backgrounds to the same level that a constitutively activated form can. Although we cannot discard a direct activation of STAT92E in response to Src and growth factors in the minority of cell types, our genetic interactions do not reveal any phenotype apart from those of the canonical JAK/STAT pathway.

Formation of STAT92E inactive dimers

In addition to the parallel dimers formed by SH2 interaction with phosphor-Tyrosine in activated STATs (Mohr et al., 2012), vertebrate STAT proteins have been shown to form ‘inactive’ antiparallel dimers mediated by the region that includes the N-terminal and the coiled-coil domains (Mao et al., 2005; Neculai et al., 2005; Ota et al., 2004). In *Drosophila*, active phosphorylated STAT92E also forms parallel homodimers through an SH2-phosphotyrosine interaction. Our study suggests that STAT92E may also form inactive homodimers mediated through the N-terminal coiled-coil domain (Fig. 2). Thus, formation

of STAT92E dimers prior to pathway activation could be an ancestral STAT characteristic, reinforcing *Drosophila* as a model for studying vertebrate STAT signalling.

STAT92E and Baz domains required for STAT92E membrane localization

We have previously described the requirement of a STAT92E-Baz interaction for efficient JAK/STAT signalling (Sotillos et al., 2008). Now, we have uncovered the domains in both molecules involved in this physical interaction. In the case of STAT92E we have narrowed down the region to the transactivation (TA) domain of the protein: only the most C-terminal part of the molecule is able to localize to the apical membrane cortex on its own (Fig. 2C) and to co-precipitate with Baz (Fig. 3A-C). Moreover, when the TA domain is removed, the C-terminal domain is unable to localize to the cell membrane where Baz is located (Fig. 1G; supplementary material Fig. S3) and reduces its ability to co-precipitate with Baz (Fig. 3C), probably owing to the loss of binding to the N-terminal part of Baz (supplementary material Fig. S4). However, this construct is still able to bind to the C-terminal part of Baz (supplementary material Fig. S4), indicating that another domain in this fragment is also involved in this interaction. In vertebrates, the TA domain has been shown to be crucial for the regulation of the activity of STAT through the interaction with several proteins (Decker and Kovarik, 1999; Horvath, 2000). Here, we have added a new function to this domain as a mediator of the interaction of STAT92E with Baz.

Baz is a scaffolding protein that is able to interact with various proteins and lipids through different regions. We have shown that interaction with STAT92E requires both the Baz N-terminal region (1-317) that includes the oligomerization domain and the C-terminal region (1048-1464) that includes the phosphatidylinositol-binding site (Fig. 3D,E; Fig. 4). Given that both Baz N- and C-terminal domains are conserved, and that STAT92E TA domain is conserved in vertebrate STATs, it is possible that the PAR3-STAT interaction is a conserved feature of JAK/STAT signalling. Our S2 cell and embryo experiments show a requirement of Src in the STAT92E-Baz interaction. Paradoxically, we are able to precipitate *STAT92E* in the absence of Src using the N- and the C-terminal fragments of Baz *in vitro*. As *in vitro* we are using Baz fragments, which most probably have a different conformation from the full-length protein, this paradox can be explained if, *in vivo*, Src function was required to change the conformation of Baz or to displace another protein that interferes with the binding, events that would not take place in the *in vitro* binding.

In summary, our results show that Src42A and Src64B are required redundantly in the ectoderm to allow STAT92E to bind to Baz. This interaction leads to the priming of JAK/STAT signalling by concentrating inactive STAT92E dimers apically near the polarized receptor kinase complex, contributing in this way to the efficient canonical JAK/STAT signalling. Although we cannot completely discard that future studies in *Drosophila* may find some specific cell type in which direct STAT92E activation by Src kinases exists, our data indicate that, in general, there is no direct STAT92E activation by Src in *Drosophila*. We would like to speculate that the existence of complexes where STAT, PAR3 and Src interact might have allowed the evolution of STAT-activation shortcuts that in vertebrates would have led to Src directly phosphorylating STAT in tyrosine 700 without the intervention of JAK or the canonical receptors. Considering the relevance of these proteins in development and disease, future studies should address whether apical STAT localization through PAR3-Src activity is also functioning in the vertebrate lineage.

Acknowledgements

We thank Erika Bach, Nicolas Tapon, Alana O'Reilly, Rebecca Spokony, Shigeo Hayashi and Tetsuya Kojima for reagents; and Andreas Wodarz, Joaquim Culí, Martin Zeidler and Sonsoles Campuzano for suggestions to the manuscript.

Funding

S.S. is a Ramón y Cajal Fellow. J.M.E.-V. is supported through grants from the Ministerio de Educación y Ciencia to J.C.-G.H. This work was supported by grants from the Programa Consolider and the Junta de Andalucía; by Ministerio de Ciencia e Innovación/Fonds Européen de Développement Régional grants to J.C.-G.H. and S.S. [BFU2010-15020 and BFU2010-15851]; and by grants from the Deutsche Forschungsgemeinschaft to M.K. [KR3901/1-1].

Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.092320/-/DC1

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