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Plasticity of *Drosophila* Stat DNA binding shows an evolutionary basis for Stat transcription factor preferences

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In vertebrates, seven signal transducer and activator of transcription (STAT) proteins bind to palindromic sites separated by spacers of two or three nucleotides (STAT1), four nucleotides (STAT6) or three nucleotides (STAT2 to STAT5a/b). This diversity of binding sites provides specificity to counter semiredundancy and was thought to be a recent evolutionary acquisition. Here, we examine the natural DNA-binding sites of the single *Drosophila* Stat and show that this is not the case. Rather, *Drosophila* Stat92E is able to bind to and activate target gene expression through both 3*n* and 4*n* spaced sites. Our experiments indicate that Stat92E has a higher binding affinity for 3*n* sites than for 4*n* sites and suggest that the levels of target gene expression can be modulated by insertion and/or deletion of single bases. Our results indicate that the ancestral STAT protein had the capacity to bind to 3*n* and 4*n* sites and that specific STAT binding preferences evolved with the radiation of the vertebrate STAT family.

Keywords: STAT; binding sites; *Drosophila*; evolution

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INTRODUCTION

The Janus kinase (JAK)/signal transducer and activator of transcription (STAT) signalling pathway was originally discovered in vertebrates on the basis of its transduction of activation of γ-interferon (Darnell, 1997; Levy & Darnell, 2002). Analysis of the pathway rapidly identified a family of seven closely related STATs as well as many pathway-activating cytokines, several receptors and four JAK kinases. The seven STAT transcription factors share several features including an SH2 domain, an invariant tyrosine residue phosphorylated as a result of the activation of STAT, and a characteristic DNA-binding domain. According to the established canonical model, activation of the JAK/STAT pathway by cytokine signalling brings about tyrosine phosphorylation of cytoplasmic STATs and leads to their dimerization. This complex translocates to the nucleus where it binds to DNA, thus activating target gene transcription (Kisseleva *et al*, 2002; Levy & Darnell, 2002). STAT DNA-binding sites, also known as Gamma interferon activation site (GAS) elements, consist of an essential core comprising the palindromic sequence TTC(*n*)GAA where *n* represents a spacer of 2–4 nucleotides. STAT6 shows a preference for 4*n* spacing, whereas other STATs preferentially bind to 3*n*, although they can also bind to 2*n* sites with low affinity (Ehret *et al*, 2001).

The Jak/Stat pathways identified in invertebrates seem to be much simpler. Of these, only *Drosophila* has a 'complete' pathway comprising three unpaired-like cytokines (Upd, Upd2 and Upd3); one receptor (Domeless; Dome); one JAK kinase (Hop) and one Stat (Stat92E; Binari & Perrimon, 1994; Hou *et al*, 1996; Yan *et al*, 1996; Harrison *et al*, 1998; Brown *et al*, 2001; Chen *et al*, 2002; Agaisse *et al*, 2003; Hombría *et al*, 2005; reviewed in Hombría & Brown, 2002; Arbouzova *et al*, 2006).

In vitro site selection and electrophoretic mobility shift assays (EMSAs) showed that *Drosophila* Stat has a binding preference for sites with 3*n* spacing (Yan *et al*, 1996). *In vivo*, Stat92E binding of 3*n* sites was confirmed for the *even skipped* (*eve*) gene (Small *et al*, 1996). In cell culture, it was also shown that *Drosophila raf* (*Draf*) and Suppressor of cytokine signalling at 36E (*Socs36E*), enhancers containing 3*n* sites are activated by Jak/Stat (Kwon *et al*, 2000; Baeg *et al*, 2005; Müller *et al*, 2005). Furthermore, vertebrate 3*n* GAS elements act as reporters for the activation of Stat in *Drosophila melanogaster* and in *Caenorhabditis elegans* (Gilbert *et al*, 2005; Wang & Levy, 2006).

These data suggest that the ancestral STAT bound to 3*n* sites and that the preference for sites with other spacing evolved after

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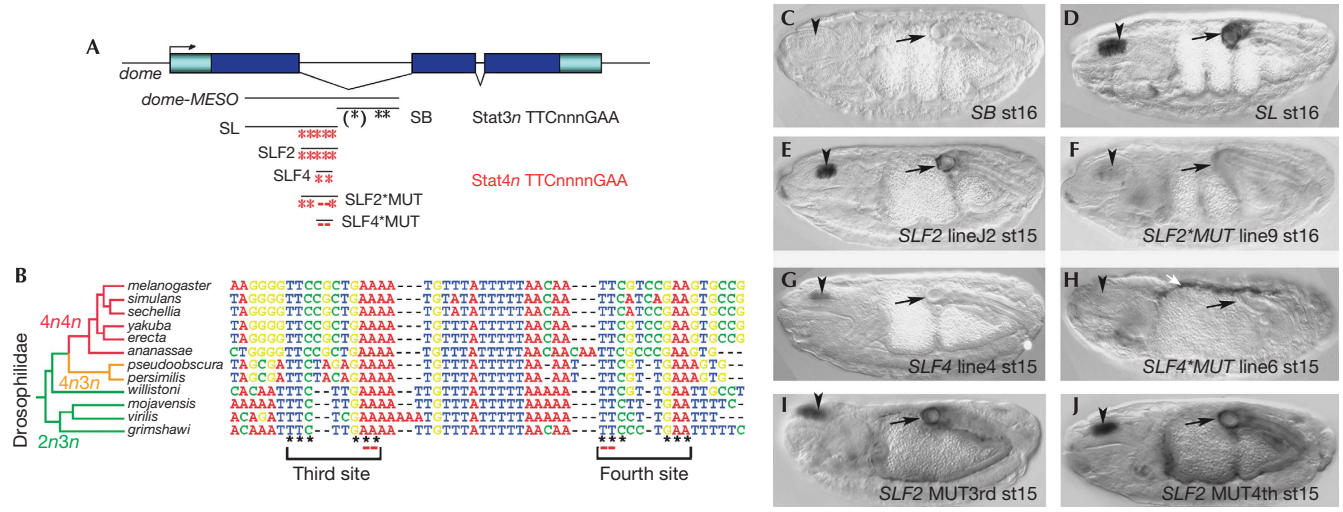

Fig 1 | Stat regulates *dome* transcription through 4*n* sites. (**A**) Schematic representation of the *dome* gene showing the localization of the putative Stat-binding sites. Sites in the first intron are represented by black (3*n*) or red (4*n*) asterisks. (**B**) Comparison of the conserved *SLF4* region in several Drosophilidae. The element has two putative Stat sites with varying spacer lengths as indicated by the tree branch colours (4*n*4*n* (red), 4*n*3*n* (orange) and 2*n*3*n* (green)). Asterisks under the sequence label the putative Stat-binding sites and red dashes indicate the mutated bases in the *SLF2*MUT* and *SLF4*MUT*. (**C**) The *SB* first intron fragment containing the 3*n* sites does not drive expression in the embryo. The *SL* (**D**) and the *SLF2* (**E**) fragments drive expression in the pharynx and hindgut. (**F**) Simultaneous mutation of the conserved third and fourth Stat-binding sites in *SLF2*MUT* abolishes most expression from the pharynx and hindgut, although low levels remain at late stages. (**G**) The *SLF4* fragment drives expression, albeit at low levels, in the pharynx and hindgut. Hindgut expression is only observed in inserts with higher levels of expression (compare **G** with Fig 2C). (**H**) Mutation of both Stat-binding sites abolishes pharynx and hindgut expression in *SLF4*MUT*. This particular line has been chosen as it has Jak/Stat-independent expression in the amnioserosa (white arrow) that acts as an internal control for staining. Mutation of only one conserved Stat site in *SLF2*, either the third site (**I**) or the fourth site (**J**), is not sufficient to abolish the expression (compare wild-type *SLF2* in (**E**) with (**I**) and (**J**) with the double mutant in (**F**)). The tree in (**B**) is modified from the assembly, alignment and annotation of 12 species as published in FlyBase. Stat, signal transducer and activator of transcription.

the vertebrate STAT radiation. Here, we present evidence that the converse is true, with binding site plasticity of STAT transcription factors representing an ancestral state. We show that *Drosophila* Stat is able to activate transcription through 4*n* sites. We show that Stat binds to 3*n* sites with higher affinity *in vitro*, and that the transformation of 4*n* into 3*n* sites increases the activation of Stat targets both *in vitro* and *in vivo*. These observations clarify how the Stat binding preferences evolved and illustrate an unanticipated plasticity of DNA binding that will help in the definition of direct Stat targets outside the vertebrate lineage.

RESULTS AND DISCUSSION

Stat92E activates *dome* through 4*n* sites

Upd expressed in the ectoderm of the *Drosophila* pharynx and hindgut signals to the adjacent mesoderm where it enhances *dome* transcription (Hombría *et al*, 2005). This effect is mediated by a mesoderm-specific enhancer (*dome-MESO*) present in the first intron of the *dome* gene (Hombría *et al*, 2005). To prove that this enhancer is regulated directly by Stat, we searched for potential Stat92E-binding sites [TTC(3*n*)GAA] in *dome-MESO* (Fig 1A; Yan *et al*, 1996) and identified three such sites at the 3′ end of the reporter. However, a 1.2-kb *lacZ* construct, *dome-SB*, containing these 3*n* sites, is unable to drive mesodermal expression (Fig 1C), whereas the complementary 1.6-kb proximal fragment, *dome-SL*, reproduces the *dome-MESO* pattern of expression (Fig 1D). The subdivision of this fragment locates the enhancer within a 746-bp fragment that we named *dome-SLF2* (Fig 1A,E). Although no canonical 3*n* sites are present in SLF2, five 4*n* sites are present—a sequence bound in vertebrates by the STAT6 protein (Ehret *et al*, 2001). As previous *in vitro* binding site selection experiments using Stat92E isolated the 3*n* sites exclusively, we set out to test whether *Drosophila* Stat92E could bind to 4*n* sites *in vivo*.

To identify which of the five potential sites drive mesodermal regulation, we compared the first intron sequence of *dome* in several Drosophilidae (Drosophila 12 Genomes Consortium, 2007). The only conserved sequence is a 43 bp element containing the third and fourth *D. melanogaster* Stat 4*n* sites (Fig 1B). To test the possible function of these two sites, we made an *SLF2* construct with the conserved third and fourth 4*n* sites mutated (*SLF2*MUT*), a 137-bp fragment containing only the two 4*n* sites present within the 43 bp conserved region (*SLF4*), and an *SLF4* construct with these two 4*n* sites mutated (*SLF4*MUT*; Fig 1B).

Analysis of *SLF2*MUT* shows that mutation of the third and fourth 4*n* sites results in the almost complete loss of mesoderm expression (Fig 1F), although low levels of expression were still observed at late embryogenesis.

The *SLF4* fragment alone is able to drive expression in the mesoderm of the pharynx and hindgut. The expression of *SLF4* is more variable than that of *SLF2* with some insertions showing expression exclusively in the pharynx (Fig 1G), whereas others showing low levels of general mesoderm expression in addition to

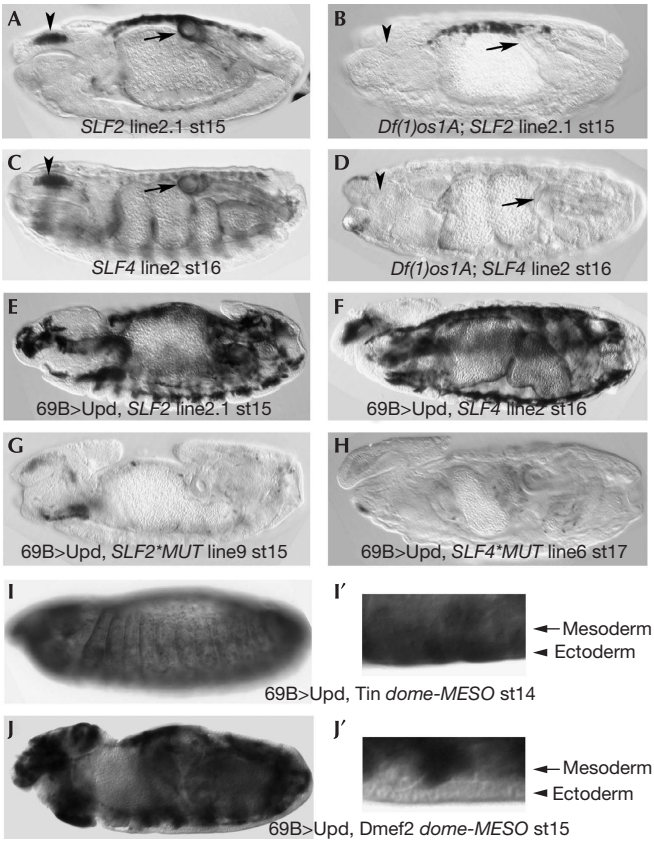

Fig 2 | Expression of *SLF2* and *SLF4* depends on Jak/Stat pathway activity. (**A**) An *SLF2* line in wild type. This insertion has expression on the amnioserosa unrelated to the enhancer. (**B**) The same insertion as in (**A**) in a *Df(1)os1A* embryo lacking all *upd* genes. The expression in the pharynx and hindgut disappears, whereas the unrelated amnioserosa staining persists, acting as an internal control for staining levels. (**C**) A wild-type *SLF4* strong insertion. (**D**) The same insertion in a *Df(1)os1A* background. (**E,F**) Ectopic *upd* in the ectoderm driven by the *69B-Gal4* line induces ectopic SLF2 (**E**) or SLF4 (**F**) expression in the mesoderm. (**G,H**) Ectopic *upd* does not induce ectopic expression when the two conserved Stat-binding sites are mutated as in *SLF2*MUT* (**G**) or in *SLF4*MUT* (**H**). (**I**) Simultaneous expression in the ectoderm of *upd* and *tin* can activate *dome-MESO* in the ectoderm. (**J**) Simultaneous expression of *upd* and *Dmef2* does not result in the activation of *dome-MESO* in the ectoderm. (**I′,J′**) Close-up views of the ventral side of the embryos in (**I,J**) showing the position of the ectoderm and mesoderm cells. Only Upd and Tin co-expression can activate the enhancer in the ectoderm. In both cases, because of the presence of the endogenous mesodermal cofactors, there is mesodermal enhancer expression. *dome-MESO*, *domeless-mesoderm-specific enhancer*; Jak, Janus kinase; Stat, signal transducer and activator of transcription.

expression in the pharynx and hindgut (Fig 2C). Mutation of the 4*n* sites in *SLF4*MUT* is sufficient to ablate all expression in both the pharynx and the hindgut (Fig 1H).

The lower levels of expression of *SLF4* relative to those of *SLF2* in the pharynx and hindgut and the slight remnant expression observed in the pharynx of *SLF2*MUT* embryos suggest that some of the non-conserved 4*n* sites might contribute to *dome-MESO* expression. We tested whether both of the conserved 4*n* sites in *SLF2* are necessary by independently mutating them (Fig 1I,J). Mutation of a single site is not sufficient to abolish mesoderm expression, indicating that these sites are redundant in the context of the SLF2 enhancer.

To confirm that *SLF2* and *SLF4* are responsive to Jak/Stat signalling, we studied their expression in *Df(1)os1A* mutants that lack all Upd ligands. As expected, *SLF2* expression disappeared from the pharynx and hindgut (Fig 2A,B) and the same is true for *SLF4* (Fig 2C,D). Conversely, ectopic activation of the pathway by expression of Upd or Upd2 using the ectoderm-specific *69B-Gal4* line activates *SLF2* (Fig 2E; data not shown) and *SLF4* (Fig 2F) in the mesoderm. This ectopic activation requires the conserved 4*n* sites as it is not observed in *SLF2*MUT* (Fig 2G) or *SLF4*MUT* (Fig 2H).

The *dome-MESO* enhancer and its derivatives are expressed specifically in the mesoderm, suggesting that Stat is interacting with tissue-specific cofactors. We tested whether *dome-MESO* could also be activated in the ectoderm if Upd was co-expressed with various mesoderm-specific proteins. We observed ectopic ectoderm expression after co-expression of Upd with Tinman (Tin; Fig 2I,I′), but not with Dmef2, Bagpipe or Biniou (Fig 2J,J′; data not shown). This suggests that Tin or one of its downstream targets is a STAT cofactor necessary for *dome* activation in the mesoderm. The requirement for this interaction explains why only the 4*n* sites in *dome-MESO* are functional.

Stat92E binds to 4*n* and 3*n* sites with different affinity

To test whether Stat92E can bind to the conserved 4*n* sites *in vitro*, oligonucleotides containing these sites (known as 4*n* Dome3 and 4*n* Dome4; Fig 3A) were used in EMSAs together with a Stat92E–GFP (green fluorescent protein) fusion protein activated by co-expression of the constitutively active Jak allele Hop^{Tuml} (Luo *et al*, 1995; Karsten *et al*, 2006). By using the established 3*n* wild-type consensus (Yan *et al*, 1996) as a positive control, a strong band is detectable (Fig 3B, arrow) that is supershifted by the addition of the GFP antibody (Fig 3B, arrowhead). The same binding conditions with the 4*n* Dome3 and 4*n* Dome4 sites also give clear band shifts that can be supershifted (and possibly stabilized) by anti-GFP (Fig 3B). However, 4*n* band shifts are considerably weaker than those produced by the 3*n* control under these *in vitro* conditions. We next tested whether 4*n* Dome sites could compete with wild-type radiolabelled 3*n* sites (3*n* wild type) for binding to activated Stat92E–GFP. Although unlabelled 3*n* wild-type-binding sites are strong competitors (Fig 3C, lanes 1–3), a 50-fold excess of mutant 3*n*-binding sites is not able to compete with labelled 3*n* wild type (Fig 3C, lane 14). By contrast, unlabelled 4*n* Dome3 and, to a lesser extent, 4*n* Dome4 sites can compete with the labelled 3*n* wild-type probe (Fig 3C, lanes 6 and 9). A Dome3 + 4 probe containing both 4*n* sites separated by their *D. melanogaster* spacer (Fig 1B) at 25-fold excess (Fig 3C, lane 12—a concentration that provides a 50-fold excess of 4*n* sites) produces stronger competition than the equivalent concentration of either 4*n* site alone, suggesting that two adjacent sites are able to bind Stat92E better than a single binding site in isolation.

To measure *in vivo* the relative transcriptional activation potential of activated Stat92E–GFP at these sites, we devised a luciferase reporter plasmid containing either four 3*n*- or 4*n*-binding sites (Fig 3D; Methods). By using these reporters in an established Kc_{167} cell-based model (Müller *et al*, 2005), we stimulated cells by

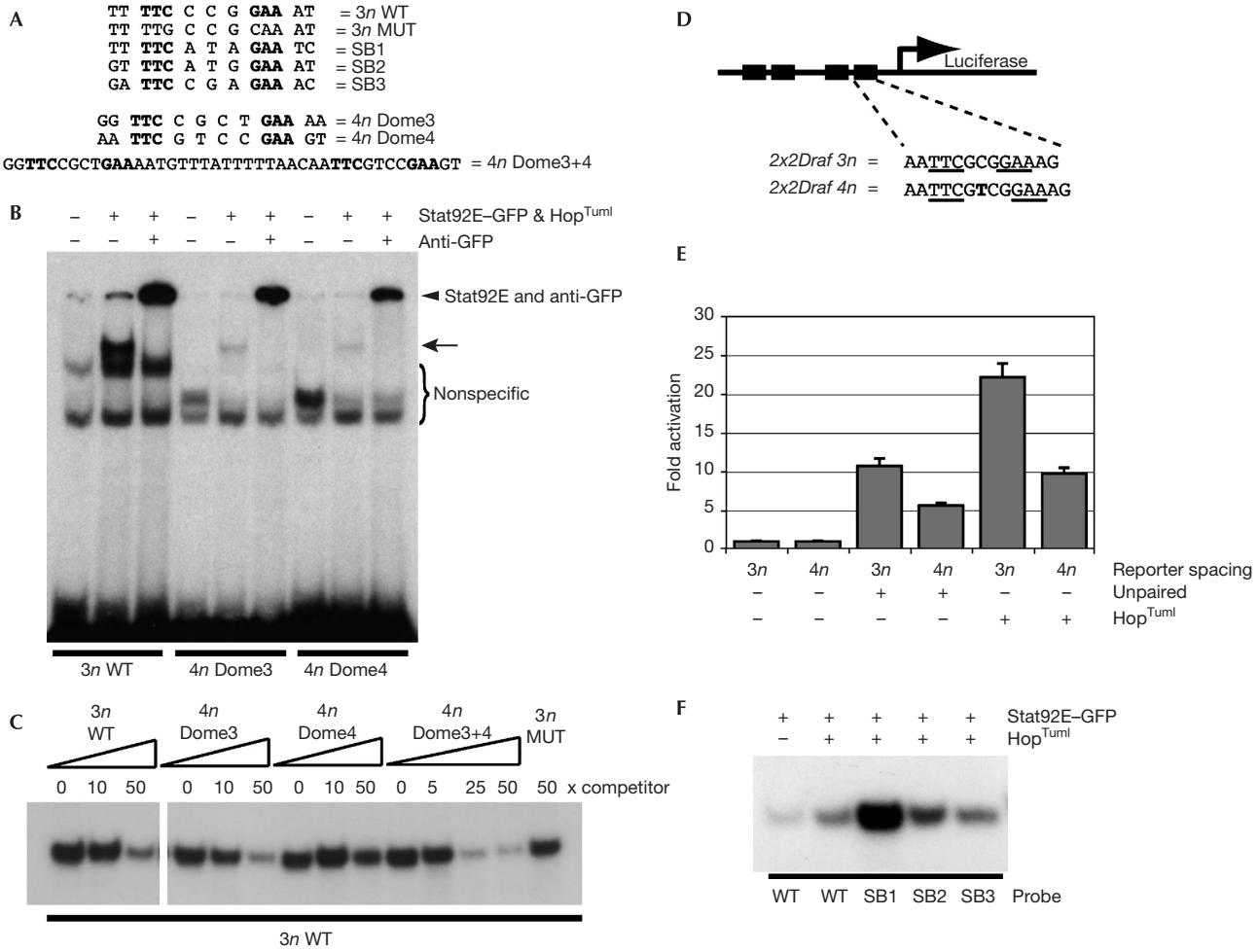

Fig 3 | *Drosophila* Stat binds to 3*n* and 4*n* sites with various affinities. (**A**) Sequences of the oligonucleotides used in (**B**,**C**,**F**). SB1, SB2 and SB3 correspond to the potential Stat-binding sites present in the SB fragment (Fig 1A), and Dome3, Dome4 and Dome3 + 4 correspond to the *D. melanogaster* Stat-binding sites in *SLF4* (Fig 1A,B). 3*n* wild type (WT) corresponds to the Stat92E-binding site consensus (Yan *et al*, 1996). (**B**) EMSA assay using the radiolabelled binding sites indicated and showing binding activities that are detectable after co-transfection of plasmids expressing Stat92E–GFP and the constitutively active Hop^{Tuml} proteins. Specific shifted bands corresponding to DNA:Stat92E–GFP (arrow) and DNA:Stat92E–GFP complexes supershifted with anti-GFP (arrowhead) are indicated. (**C**) EMSA assay using radiolabelled 3*n* WT to detect activated Stat92E–GFP-binding activity. Each lane contains the same quantity of cell extract and labelled 3*n* WT-binding site and was co-incubated with the indicated fold excess of unlabelled competitor sites. (**D**) Schematic representation of the reporters used in (**E**) with black boxes representing Stat-binding sites. Underlined bases represent the core Stat92E-binding sequence. Another thymine residue (bold) was inserted into each binding site of *2x2Draf 3n* to create the *4n* reporter. (**E**) Firefly luciferase activity in cells transfected with the reporters shown in (**D**) and co-transfected with plasmids expressing either the pathway ligand unpaired or the constitutively active Jak Hop^{Tuml}. Levels were normalized to a co-transfected constitutively expressed *Renilla* luciferase plasmid and are expressed as fold change over unstimulated state. (**F**) EMSA assay of unactivated and activated Stat92E–GFP binding to radiolabelled WT, SB1, SB2 and SB3 oligonucleotides. EMSA, electrophoretic mobility shift assay; GFP, green fluorescent protein; MUT, mutated; STAT, signal transducer and activator of transcription.

either co-expressing the Upd ligand or the activated Jak Hop^{Tuml} (Luo *et al*, 1995). Under these conditions, both 3*n* and 4*n* reporters show significantly increased levels of activity over the unstimulated state, with the 3*n* reporter around twice as active as the 4*n* reporter (Fig 3E). Finally, we found that the *SB* 3*n* sites (Figs 1A,3A) can bind to Stat92E *in vitro* (Fig 3F), underscoring the importance of cofactors for the activity of Stat *in vivo*.

Comparison of the conserved SLF4 region in various Drosophilidae (Fig 1B) shows that species closely related to *D. melanogaster* share the 4*n* spacing of the Stat fourth site, with 3*n* sites present in more distantly related drosophilids. In some species in which the fourth site is 3*n*, the third site has a 2*n* spacer. The above experiments suggest that changes in spacer length during evolution might modulate the levels of transcription of target genes. To test this possibility, we mutated the fourth 4*n* spacer in *SLF4* to a 3*n* spacer as observed in the *obscura* group. The resulting enhancer drove higher levels of expression in *D. melanogaster* than did *SLF4* (Fig 4A,B), indicated by the

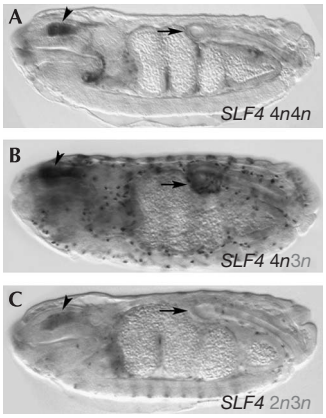

Fig 4 | Various Stat spacer lengths modify *in vivo* transcriptional activation. (**A**) Levels of expression of the *SLF4* enhancer containing the normal 4*n*4*n* *D. melanogaster* Stat-binding sites. (**B**) Enhanced expression of an *SLF4* enhancer in which the conserved fourth Stat 4*n* site is transformed to 3*n*. (**C**) An *SLF4* enhancer in which the third site has been transformed to 2*n* and there is a 3*n* site transformation on the fourth site. Note that the levels of expression in (**C**) are lower than those in (**B**), and more similar to those of (**A**). Stat, signal transducer and activator of transcription.

consistent appearance of hindgut expression in all insertions. Further mutation in this enhancer of the third-site to a 2*n* spacer, as in *D. virilis,* restored the levels of expression similar to the original *SLF4* enhancer (Fig 4C). These results indicate that varying the relative number of 3*n* compared with 4*n* sites might control the level of expression of Stat targets during evolution.

The direct Stat target *crb* is regulated through 4*n* sites

To determine whether other *Drosophila* Stat target genes are controlled through 4*n* sites, we analysed the Stat-dependent *crb* spiracle-specific enhancer (Fig 5A,B; Lovegrove *et al*, 2006). Simultaneous mutation of the three putative Stat-binding sites (one 3*n* and two 4*n* sites) reduces *crb* spiracle expression (Fig 5C). Mutation of the 3*n* site has little effect on the expression of enhancer (Fig 5D), whereas mutation of both 4*n* sites (Fig 5E) results in levels of expression similar to those obtained after mutation of both the 3*n* and 4*n* sites.

Our results highlight the limitations of bioinformatic and *in vitro* DNA-binding analyses as sole methods for defining transcription-factor-binding sites if they overlook low-affinity binding sites that might be functional *in vivo* because of tissue-specific cofactors.

The capacity of *Drosophila* Stat to activate through both 3*n* and 4*n* sites suggests that the ancestral Stat protein had the ability to bind to both sites. This capacity has been retained in *Drosophila* and possibly in other invertebrates. Intriguingly, this suggests that the *Drosophila* Stat protein dimer has some flexibility in its ability to bind to DNA sites with different spacing. The loss of this flexibility after the radiation of the vertebrate STATs might have resulted in the differential 3*n* compared with 4*n* binding preferences observed in mammalian STAT proteins. In vertebrates, the specialization of various STAT proteins for different binding sites might have been advantageous for acquiring target specificity (Seidel *et al*, 1995), although it is possible that vertebrate STATs

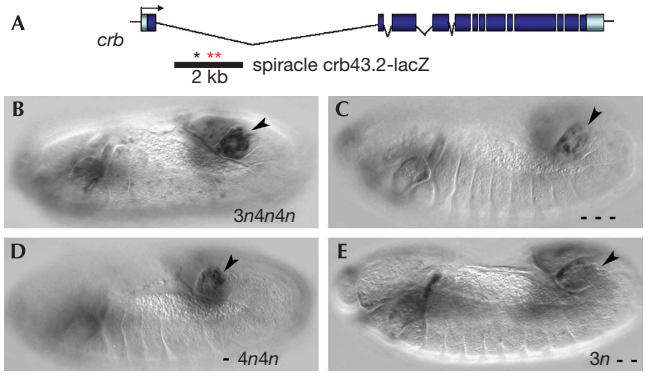

Fig 5 | The *crb* gene is regulated by Stat through 4*n* sites. (**A**) Schematic representation of the *crb* gene showing the localization in the spiracle enhancer of the putative Stat-binding sites analysed (red asterisks (4*n* sites), black asterisk (3*n* site)). (**B**) Expression of the spiracle enhancer in the wild type. (**C**) Expression in the triple 3*n*4*n*4*n* mutant (- - -). (**D**) Expression in the 3*n* mutant site (-4*n*4*n*). (**E**) Expression in the double 4*n*4*n* mutant sites (3*n*- -). Stat, signal transducer and activator of transcription.

can also activate targets through low binding affinity sites. In *Drosophila,* in which it has been shown that Upd acts as morphogen (Xi *et al*, 2003), maintaining Stat binding flexibility might be advantageous, as the evolution of the number of Stat-binding sites and their spacer length could provide a flexible system to modulate the distance from the source at which a given Stat target could be activated.

METHODS

Constructs and directed mutagenesis. All reporter constructs were generated in *phs43lacZ*. To create the SB and SL reporters, the *dome-MESO* 2.8-kb enhancer fragment (Hombría *et al*, 2005) was subdivided into a distal (SB) *Eco*RV-*Bam*H1 and a proximal *Not*I-*Eco*RV (SL) fragment. PCR amplification of the SL fragment with the primers TAGGAGGGGAACTGGGATGG and ATGTTT GGCCTCGAAATTGC generated 746 bp SLF2. Amplification with CGAATACGTTAGGGCGAGCC and GTACATCGGCACTTCGG ACG created 138 bp SLF4. Amplified fragments were subcloned into *pGEMT* and from there into *phs43lacZ*.

The conserved Stat sites in SLF4 and SLF2 were *in vitro* mutagenized into TTCCGCTGTT, the third site, and AACGTCC GAA, the fourth site (Fig 1B), using QuikChange (Stratagene; www.stratagene.com) and appropriate PAGE-purified primers to create SLF2*MUT and SLF4*MUT. These sites were mutated independently in SLF2 to create SLF2MUT3rd and SLF2MUT4th. The same sites were also mutated to create various spacer variants of SLF4. In SLF4 4*n*3*n*, the fourth site was mutated to TTC.TCCGAA where (.) indicates a deleted G. In SLF4 2*n*3*n*, apart from this deletion, the third site was mutated to TTCC..TGAA where (..) indicates deleted GC.

The wild-type crb43.2 enhancer and the triple mutant Stat-binding-site spiracle enhancer have been described earlier (Lovegrove *et al*, 2006). The mutations in the triple mutant (- - -) were TTCCATGCC (for 3*n*), TTCGTTTGTT (for 5′ 4*n*) and TTCAGGGGTT (for 3′ 4*n*). In the 3*n* mutated construct (-4*n*4*n*), the 3*n* site was TTCCATGTT. In the double 4*n* mutant construct

(3*n*--), the sites were transformed to TTCGTTTGTT (5′ 4*n*) and TTCAGGGGTT (3′ 4*n*). For each construct, several independent inserts were analysed using anti-β-galactosidase. For SLF4, SLF2*MUT and SLF4*MUT, 10 inserts were studied in each case to confirm that the expression was consistent. All fly strains have been described by Hombría *et al* (2005). *Df(1)os1A* is a deletion for all three Upd ligands (Upd, Upd2 and Upd3).

Electrophoretic mobility shift assays. EMSAs to detect DNA binding of Stat92E were undertaken as described by Karsten *et al* (2006). Double-stranded DNA probes were generated by annealing GGAGGGTTCCGCTGAAAAT and GACATTTTCA GCGGAACCC (4*n* Dome3), GGACAATTCGTCCGAAGTG and GACCACTTCGGACGAATTG (4*n* Dome4), GGAGGGTTCCGCT GAAAATGTTTATTTTTAACAATTCGTCCGAAGTG and GACC ACTTCGGACGAATTGTTAAAAATAAACATTTTCAGCGGAACCC (4*n* Dome3 + 4), GGAATTTTCATAGAATCA and GACTGATTC TATGAAAAT (3*n* SB1), GGAGGTTTCATGGAAATC and GAC GATTTCCATGAAACC (3*n* SB2), GGACGATTCCGAGAACTG and GACCAGTTCTCGGAATCG (3*n* SB3), GGATTTTTCCCGGAA ATG and GACCATTTCCGGGAAAAA (3*n* wild type) or GGATTT TTGCCGCAAATG and GACCATTTGCGGCAAAAA (3*n* MUT). The recessed ends of annealed oligonucleotide pairs were filled in using Klenow polymerase and dNTPs containing either ^{32}P-γ-dCTP (to generate radioactive probes) or unlabelled dNTPs (to generate cold competitors). EMSAs shown in Fig 3B,F used 0.15 pmol of radiolabelled probe per lane and 50 μg ml^{-1} poly dI-dC, whereas supershifts included 0.3 μl α-GFP antibody (Abcam; www.abcam.com). For competition assays (Fig 3C), binding to 0.06 pmol of radiolabelled 3*n* wild-type probe was assayed in the presence of either 0, 0.6 or 3 pmol of unlabelled Dome3, Dome4, 3 pmol 3*n* MUT or 0.3, 1.5 or 3 pmol of Dome3 + 4 added as competitor with 83 μg ml^{-1} poly dI-dC.

Luciferase assays. The *2x2Draf 3n* luciferase reporter is based on the p5′-663*Draf*wt-*luc* originally containing two 3*n* Stat-binding sites (Kwon *et al*, 2000) that we duplicated to generate a reporter containing four 3*n* sites. We also generated another version of p5′-663*Draf*wt-*luc in vitro* which was mutated to include another spacer nucleotide that we duplicated to generate a reporter containing four 4*n* sites (known as 2x2Draf 4*n*).

Transformation of the 3*n* to 4*n* sites was generated *in vitro* using the QuikChange method (Stratagene) and the oligonucleotides GGGGATCCTAAAATTCG**T**CGGAAAGTAATAAAATTCG**T** CGGAAAGTAAAGATCCCCCG and CGGGGGATCTTTACTTTC CG**A**CGAATTTTATTACTTTCCG**A**CGAATTTTAGGATCCCC (underlined bases represent the core Stat92E-binding sequence and bold indicates the added base). Both the original 3*n* and the newly generated 4*n* vectors were then multimerized by cutting out the binding sites using *Bam*HI and *Xba*I, filling in and re-ligating into the parental vector cut *Sma*I to generate 2x2Draf 3*n* and 2x2Draf 4*n*, respectively. Constructs were sequence verified. Activity assays were undertaken in Kc_{167} cells as described by Müller *et al* (2005). Equal quantities of both 3*n* and 4*n* reporters were transfected for each experiment repeated in quadruplicate and normalized to unstimulated background activity.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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