Differential *in vivo* requirements for oligomerization during Groucho-mediated repression

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The Groucho (Gro)/transducin-like enhancer of split family of transcriptional corepressors are implicated in many signalling pathways that are important in development and disease, including those mediated by Notch, Wnt and Hedgehog. Here, we describe a genetic screen in *Drosophila* that yielded 50 new *gro* alleles, including the first protein-null allele, and has two mutations in the conserved Q oligomerization domain that have been proposed to have an essential role in corepressor activity. One of these latter mutations, encoding an amino-terminal protein truncation that lacks part of the Q domain, abolishes oligomerization *in vitro* and renders the protein unstable *in vivo*. Nevertheless, the mutation is not a null: maternal mutant embryos have intermediate segmentation phenotypes and relatively normal terminal patterning suggesting that the mutant protein retains partial corepressor activity. Our results show that homo-oligomerization of Gro is not obligatory for its action *in vivo*, and that Gro represses transcription through more than one molecular mechanism.
Keywords: *Drosophila*; Groucho; repression; segmentation; transcription

INTRODUCTION

Groucho (Gro)/transducin-like enhancer of split (TLE) proteins are recruited to act as corepressors for many different families of transcription factors, including RUNX, Nkx, Hes and LEF1/TCF, and are crucial in many developmental signalling pathways, including Notch and Wnt (Chen & Courey, 2000; Gasperowicz & Otto, 2005). Gro is required in *Drosophila* development for many processes, including terminal patterning, segmentation, sex determination, dorsal–ventral patterning and neurogenesis (reviewed by Chen & Courey, 2000). In vertebrates, Gro/TLE proteins participate in a diverse range of processes, including neurogenesis, osteogenesis and haematopoiesis (reviewed by

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Gasperowicz & Otto, 2005), and have been implicated as oncogenes in lung cancer (Allen *et al*, 2006).

Gro/TLE proteins contain five discernible domains: Q (oligomerization and protein interactions), GP (repression), CcN (nuclear localization), SP (repression) and WD (protein interactions, repression), of which the first and last are the most highly conserved (Stifani *et al*, 1992; Chen & Courey, 2000). The WD domain folds to form a β-propeller (Pickles *et al*, 2002), which mediates protein–protein interactions with several repressors, including Hairy (through a carboxy-terminal WRPW peptide motif) and Engrailed (through the 'eh1' motif; Paroush *et al*, 1994; Fisher *et al*, 1996; Jimenez *et al*, 1997; Jennings *et al*, 2006).

Sequences within the glutamine-rich Q domain are predicted to form two amphipathic α-helical motifs—AH1 and AH2—that facilitate oligomerization of Gro/TLE molecules into tetramers *in vitro* (Pinto & Lobe, 1996; Chen *et al*, 1998; Song *et al*, 2004). Previous studies have indicated that oligomerization of the Q domain is required for Gro to function as a corepressor in cultured cells, and for ectopic Gro to cause developmental defects in the fly (Song *et al*, 2004). The Q domain also interacts with several repressors, including LEF1/TCF during Wnt signalling (Daniels & Weis, 2005).

Here, we describe the recovery and characterization of many new *Drosophila gro* alleles, including a null allele and two mutations that disrupt the Q domain. One of the latter mutations—*gro*[MB12]—leads to an amino-terminal truncation that abolishes oligomerization. Gro[MB12] protein retains significant activity *in vivo*, indicating that homo-oligomerization of Gro is not essential for all its corepressor activities.

RESULTS AND DISCUSSION

Isolation of new *gro* alleles

The Hairy basic helix–loop–helix (bHLH) protein is a transcriptional repressor that depends on maternally contributed Gro to establish spatial periodicity during *Drosophila* embryogenesis. Hairy is expressed in stripes in the unicellular blastoderm embryo in which it, in turn, establishes complementary stripes of one of its target genes, *fushi tarazu* (*ftz*; Howard & Ingham, 1986). Hairy also contributes to progressive retinal differentiation in the imaginal disc of the eye (Brown *et al*, 1991).
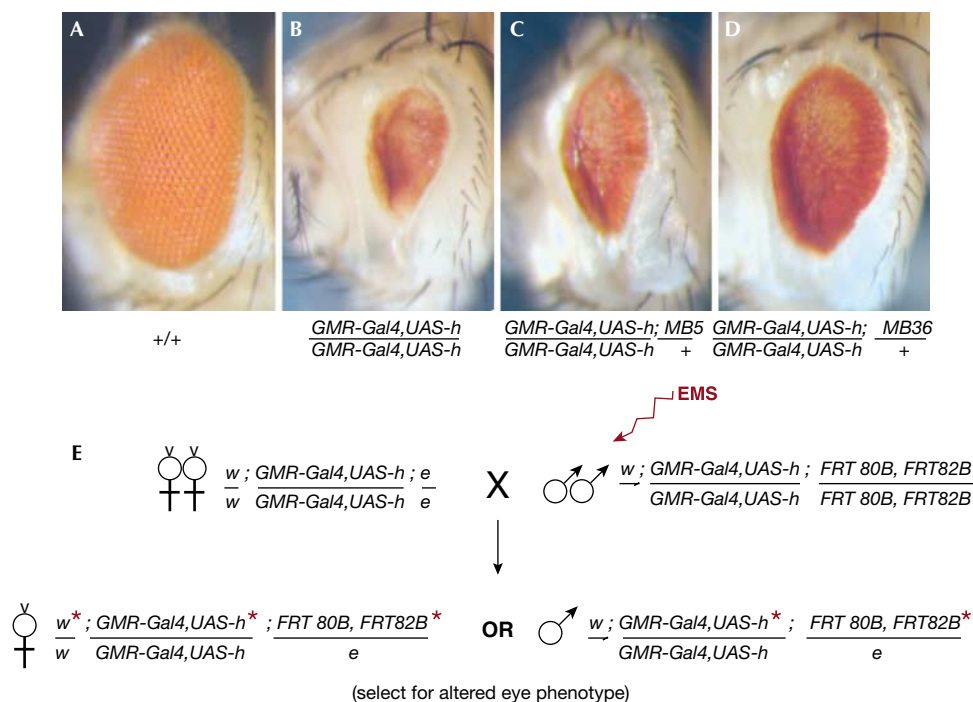
To identify new factors that interact with Hairy *in vivo*, we established a genetic screen for mutations that modulate the

Fig 1 | Genetic screen for modifiers of ectopic *hairy* expression in the eye of *Drosophila*. (**A–D**) Light photomicrographs showing dominant suppression of the eye phenotype by *gro* alleles; weak for *MB5* and strong for the null allele *MB36*. Genotypes are as indicated below. (**E**) Schematic representation of the screening strategy. F$_1$ progeny homozygous for *GMR-Gal4,UAS-hairy* and heterozygous for a mutagenized *FRT 80B FRT 82B* chromosome were scored for the suppression of the eye phenotype. *e*, *ebony*; EMS, ethyl methane sulphonate; Gro, Groucho; *h*, *hairy*; *w*, *white*.

effects of ectopic Hairy expression in the developing eye (Fig 1). Driving Hairy expression in cells posterior to the morphogenetic furrow (*GMR-GAL4;UAS-hairy*; Hay *et al*, 1994) led to a marked reduction in the size of the eye and some loss of pigmentation (Fig 1B). A pilot screen for ethyl methane sulphonate-induced modifier mutations showed that a high proportion of the suppressor mutations on chromosome 3 were allelic to *gro* (supplementary information online).

From approximately 137,000 mutated chromosomes, we recovered 62 suppressors that mapped to chromosome 3, of which 50 were new *gro* alleles. Single-stranded conformation polymorphism analysis and DNA sequencing showed specific molecular lesions in 22 alleles (Table 1; supplementary information online). Most of the *gro* mutations represent nonsense or frameshift alleles encoding truncated Gro proteins.

Seven of the *gro* mutations are missense mutations that largely abolish Gro activity and all lie within the WD domain (Jennings *et al*, 2006). The intensive screen led to the recovery of independent, identical mutations at four different sites (Table 1). Nevertheless, no missense mutations were recovered in the central GP, CcN or SP domains of Gro, suggesting that these domains are functionally dispensable *in vivo* or represent very small mutagenic targets.

We also recovered a protein-null allele—*gro^MB36*—and two alleles containing deletions within the Q domain: *gro^MB5* contains a small in-frame deletion, whereas *gro^MB12* is a single-base-pair substitution in the initiator codon that encodes an N-terminal truncated protein. We describe these in more detail below.

gro^MB36 is a protein-null allele

MB36, and the independently recovered *MB15* allele, represents a G–A mutation at position 101 in the first intron that generates an ectopic splice acceptor site. Sequencing *MB36* complementary DNA shows that they include 31 bp of intronic sequences and thereby encode only the first 12 amino acids of Gro, with the following 104 amino acids being derived from intronic and frameshifted sequences (Table 1; Fig 2A). MB36 lacks all conserved Gro protein domains and should be inactive and probably unstable. No Gro protein was detected in *MB36* embryos (embryos that were derived from homozygous *MB36* germ cells thus contain only mutant protein; Fig 2C), although this might be because the truncated protein lacks an appropriate epitope. *MB36* transcripts are indeed mis-spliced and under-represented fivefold, presumably owing to nonsense-mediated messenger RNA decay (supplementary information online). We conclude that *MB36* is a null allele of *gro*.

Maternal *MB36* embryos show severe cuticle loss owing to ectopic neurogenesis at the expense of epidermal development (Fig 3D). This 'neurogenic' phenotype is associated with the inability of the E(spl) bHLHs, targets of Notch signalling, to repress proneural transcription in the absence of Gro (Fig 3X; Paroush *et al*, 1994). Other patterning events are also affected in *MB36* embryos such that their phenotype resembles that previously described for *gro^E48* embryos (see below).

Q domain mutations retain partial function

The two new *gro* alleles with lesions within the Q domain allowed us to study the roles of this domain *in vivo*. *MB5* includes a

Table 1 | *groucho* alleles for which molecular data were obtainable

Type of lesion	Allele	Base change*	RNA/protein changes
Nonsense			
	MBB	G4624A	W550stop
	MB6	C2315T	R190stop
	MB11	C4769T	Q599stop
	MB25	C4361T	Q463stop, as *MB27*
	MB27	C4361T	Q463stop, as *MB25*
	MB34	G4749A	W592stop
	MB42	G4716A	W581stop
Frameshift			
	MB13	G2485A	Splice junction mutated, frameshift from residue 225 as intronic sequences transcribed, as *MB37*
	MB15	G138A	Ectopic splice acceptor site in intron, frameshift from residue 13, as *MB36*
	MB18	G266A	Splice junction mutated, frameshift from residue 45 as intronic sequences transcribed
	MB33	Δ3782–3813	31 bp deletion; frameshift from residue 103
	MB36	G138A	as *MB15*
	MB37	G2485A	as *MB13*
Other			
	MB5	T189C	P19S, Δ19–27
		C190T	See Fig 2B for sequence
		Δ191–214	
	MB12	G3A	Initiator ATG→ATA
Missense†			
	MBD	A4613T	I547F
	MB19	G4683A	C570Y
	MB21	G4422A	R483H, as *MB41*
	MB30	A4977C	H646P
	MB31	C5176T	L692F
	MB35	G4326A	G451D
	MB41	G4422A	R483H, as *MB21*

*Bases numbered from the start of translation, corresponding to 3R:21869066 *Drosophila melanogaster* genome Release 5.1.
†For characterization of missense alleles, refer to Jennings *et al* (2006).

complex deletion and inversion that leads to an 8-amino-acid deletion/1-amino-acid substitution lacking the first four amino acids of the AH1 helix (Table 1; Fig 2A,B). *MB5* embryos show loss of alternate ventral denticle bands, resembling *hairy* mutant embryos (Fig 3B; Howard & Ingham, 1986). However, the segmentation phenotype is weak: usually only 2–3 even-numbered denticle belts are missing (0% wild type, 5% missing one belt, 27% missing two belts, 52% missing three belts, 16% missing more than three belts; $n = 110$), and expression of *ftz* is only slightly perturbed (Fig 3N). *MB5* embryos seem to be otherwise normal (Fig 3F,J,R,V; data not shown); however, the lesion must disrupt an essential Gro activity because homozygous and hemizygous adults were never recovered.

The second Q domain mutation, *MB12*, is a single-base-pair substitution in the initiator ATG codon. MB12 protein migrates slightly faster than Gro⁺ on denaturing gels (Fig 2C), indicating that it is an N-terminal truncated protein lacking the complete AH1 amphipathic α-helix, in which translation is initiated at the next in-frame start codon, 58 codons downstream (Fig 2A; Chen *et al*, 1998; Song *et al*, 2004).

N-terminal truncation clearly disrupts Gro activity. *MB12* embryos have a moderate to strong hypomorphic neurogenic phenotype, as judged by the lack of embryonic cuticle and by ectopic expression of the Hunchback (Hb) neural marker in stage 12 embryos (Fig 3C,W). The mutation abolishes terminal repression of *snail* (*sna*) transcription by Huckebein (Hkb), so that *sna* is expressed ectopically at the termini of the embryo, as in *MB36* and *E48* embryos (Fig 3S,T; Goldstein *et al*, 1999).

Gro oligomerization has been implicated in the repression of the *vgQ-lacZ* reporter by Brinker (Brk; Hasson *et al*, 2001); thus, *vgQ-lacZ* is inhibited by overexpression of Gro⁺, but not by the oligomerization-defective mutation Gro^{L38D,L87D} (Song *et al*, 2004). We found that *vgQ-lacZ* transcription was upregulated in null (*MB36*) clones at the anterior and posterior edges of the wing pouch, as previously reported for the *E48* allele (Fig 4A,B; Hasson *et al*, 2001), showing that *vg* is indeed a target of Gro-mediated repression. *vgQ-lacZ* expression was also upregulated in *MB12* clones, but not in *MB5* clones (Fig 4C,D), indicating that MB12 is unable to support Gro-mediated repression by Brk in the wing imaginal disk.

Nevertheless, MB12 maintains repressive activity in some contexts. Mutant embryos retain more body mass than null *MB36* embryos (Fig 3C,D), and the broadened *ftz* stripes retained distinct periodicity, unlike *gro* null mutant embryos in which all *ftz* stripes are completely merged (Fig 3O,P; Paroush *et al*, 1994). More strikingly, Gro-mediated terminal repression is almost normal in *MB12* embryos. *tailless* (*tll*) expression is restricted to the ends of wild-type embryos by the Gro-dependent repressor Capicua (Cic), thereby allowing expression of *knirps* (*kni*) and *Krüppel* (*Kr*) in a central domain (Paroush *et al*, 1997; Jimenez *et al*, 2000). A total of 93% of the *MB12* embryos showed wild-type or only slightly expanded *tll* expression ($n = 22$; Fig 3G), and most mutant embryos still expressed the gap genes *kni* (96%; $n = 45$) and *Kr* centrally, although at reduced levels (Fig 3K; data not shown). By contrast, Cic activity is completely lost in *MB36*, leading to expanded *tll* expression and loss of expression of the central domains of *kni* and *Kr* (Fig 3H,L; data not shown).

Levels of Gro protein in *MB12* embryos are greatly reduced ($<5\%$ of wild type; Fig 2C; supplementary information online). *MB12* transcripts are translated as efficiently as wild type *in vitro* (Fig 5A,E,F), indicating that the low *in vivo* levels of MB12 probably resulted from protein instability. Reduced protein levels might also contribute to the *MB12* phenotype, reinforcing the view that the N-terminal truncated protein retains significant WRPW-mediated repressive activity.
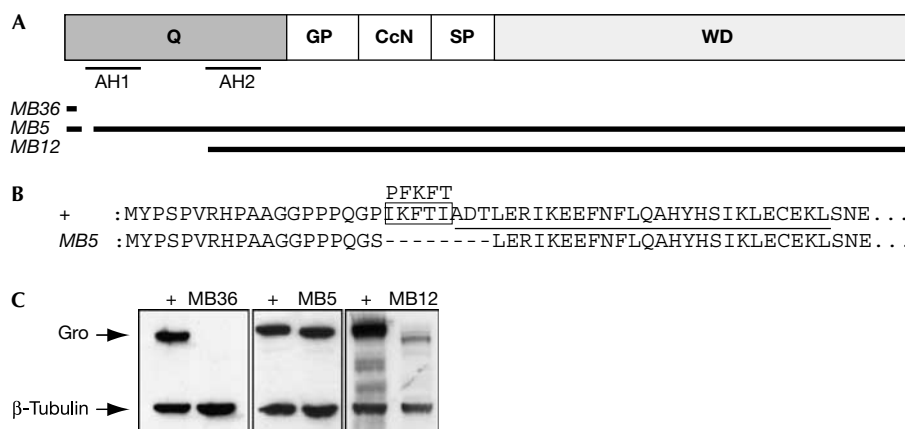
Fig 2 | Deletions recovered in *gro*. (**A**) Diagram of the domain structure of *Drosophila* Gro. The two predicted amphipathic α-helices in the Q domain (AH1 and AH2) are indicated. The coding regions of *MB36*, *MB5* and *MB12* transcripts are also shown. (**B**) Alignment of the amino-acid sequences of the mutated region in *MB5* to the corresponding wild-type region (+). AH1 is underlined; amino-acid residues deleted in *MB5* that lie outside AH1 are boxed with the corresponding sequence from hTLE1 shown above. (**C**) Western blots of extracts from *gro* mutant embryos (from *gro* mothers) run on SDS–polyacrylamide gel electrophoresis gels. MB36 is not detected and levels of MB12 are reduced. β-Tubulin is shown as a loading control. Gro, Groucho.
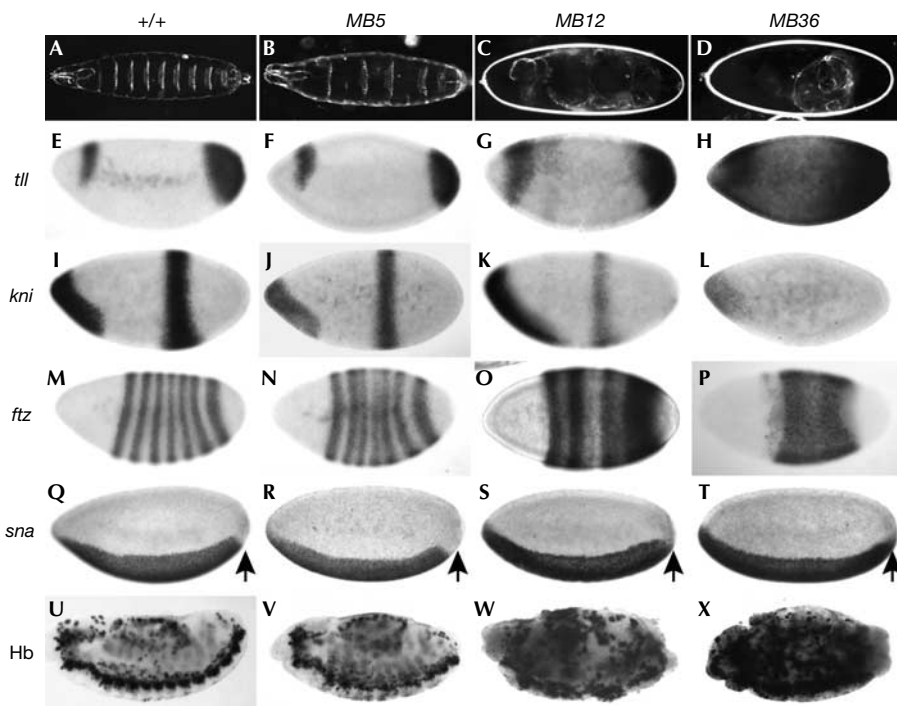
Fig 3 | Phenotypic analysis of *gro* alleles. (**A–D**) Cuticles of wild-type (+) and *gro* mutant embryos. *MB5* embryos show a weaker phenotype (loss of two or three even-numbered denticle belts) than *MB12* or *MB36*. *MB12* embryos are neurogenic but retain more body mass than *MB36*. Expression of (**E–H**) *tll*, (**I–L**) *kni* and (**M–P**) *ftz* transcripts in blastoderm embryos, showing that MB5 and MB12 retain activity. *tll* expression is wild type in *MB5*, slightly expanded in *MB12* and throughout the embryo in *MB36*, indicating residual activity in *MB12*. Expression of the central stripe of *kni* is reduced in most *MB12* embryos, but not completely abolished as in *MB36*. Stripes of *ftz* expression are irregular in *MB5*, broadened in *MB12* and completely fused in *MB36*. (**Q–T**) Expression of *sna* transcripts is expanded towards the posterior pole in *MB12* or *MB36* embryos (indicated by arrows) in a manner resembling the *sna* expression pattern in *hkb* mutants (Goldstein *et al*, 1999), indicating that repression mediated by Hkb is lost in *MB12* embryos. (**U–X**) Staining of stage 13 wild type and *gro* for the neural marker Hb. *MB12* and *MB36* mutants show ectopic expression of Hb protein that is characteristic of excessive neural development. *ftz, fushi tarazu*; Gro, Groucho; Hb, Hunchback; Hkb, Huckebein; *kni, knirps*; *sna, snail*; *tll, tailless*.
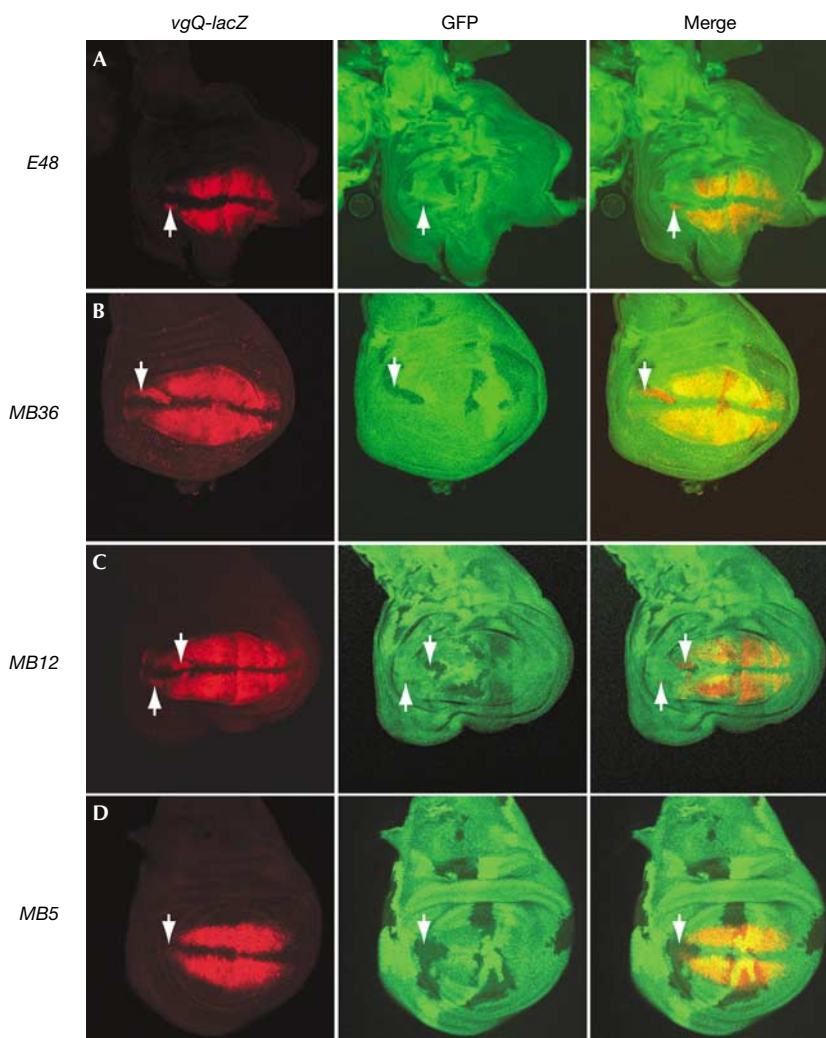
Fig 4 | Expression of *vgQ-lacZ* in *gro* mutant clones. Third instar imaginal wing disks (anterior to the left and dorsal to the top) stained for GFP and β-galactosidase. Loss of GFP (green) marks clones of homozygous *gro* mutant cells. Ectopic *vgQ-lacZ* expression is detected in clones of (**A**) *E48*, (**B**) *MB36* and (**C**) *MB12* at the anterior and posterior edges of the wing pouch but not in clones of (**D**) *MB5*. Arrows indicate positions of *gro* clones at the edge of the wing pouch; GFP, green fluorescent protein; Gro, Groucho.
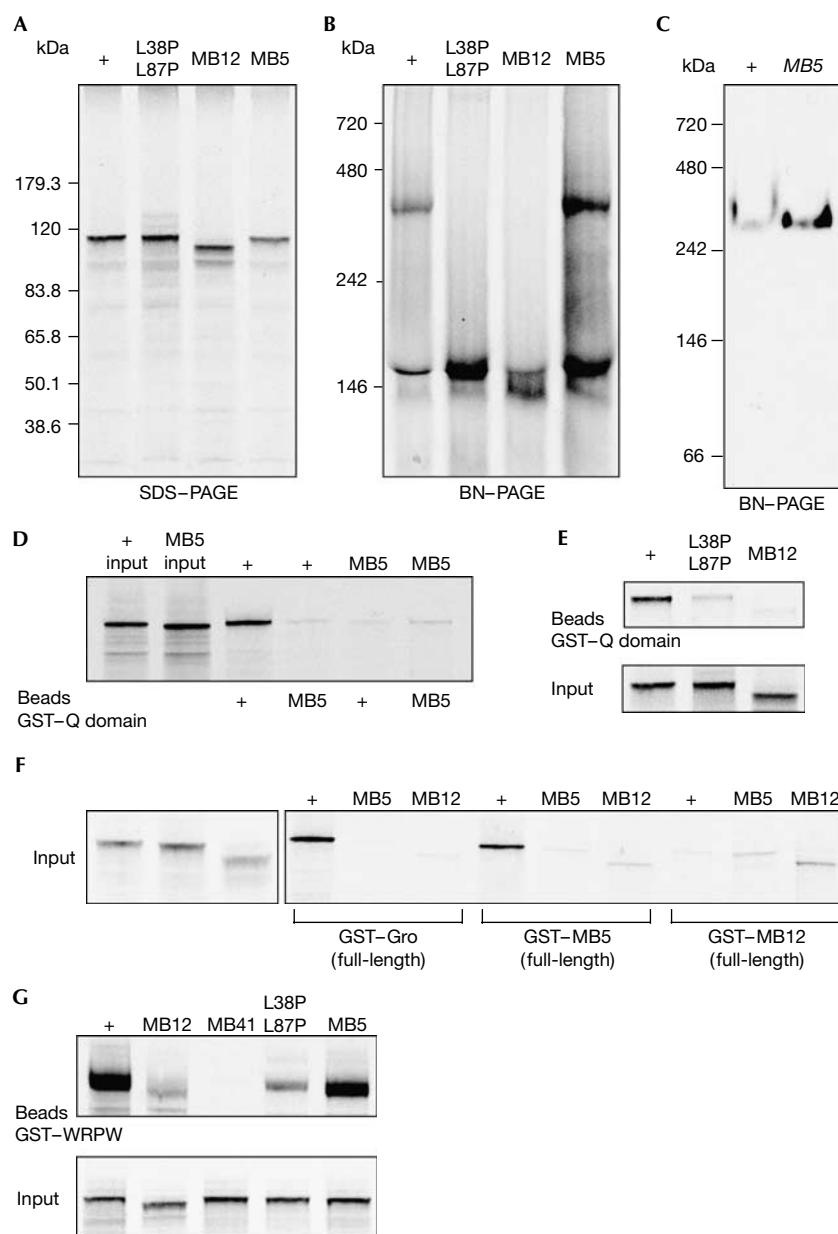
MB12 abolishes Q-domain-mediated oligomerization

The residual activity of MB5 and MB12 suggests that Gro oligomerization is not absolutely required for Gro corepressor activity. We tested whether these mutations are still able to oligomerize by measuring binding of [³⁵S]Gro to glutathione-*S*-transferase (GST) fusions of full-length Gro or to the Q domain alone (GST–Q). We also used blue-native polyacrylamide gel electrophoresis (BN-PAGE) to visualize oligomerization of translated and embryonic full-length Gro (supplementary information online). This latter assay shows two main bands of *in vitro*-translated Gro⁺: a fast-migrating, monomeric band and a slow-migrating band that represents a Gro tetramer as indicated by its molecular weight and its absence from a non-oligomerizing Gro mutant, Gro^L38P,L87P (Fig 5B; Chen *et al*, 1998).

These assays show that the short deletion in *MB5* reduces but does not abolish Q domain dimerization. GST–Q^MB5 binds to neither translated Gro⁺ nor MB5 protein (Fig 5D), and GST–Gro

(full-length) is unable to bind to [³⁵S]MB5 (Fig 5F). However, GST–Gro^MB5 (full-length) still binds to [³⁵S]Gro⁺ (Fig 5F), showing that the binding activity is not completely lost. Indeed, native gel assays show a band of tetrameric MB5 both after *in vitro* translation (Fig 5B) and also *in vivo*, as detected by western blot analysis of extracts from maternal *MB5* embryos (Fig 5C).

These results are consistent with previous analysis of Gro oligomerization *in vitro*, in which mutating an individual amino acid mediating contact between the two AH domains is insufficient to abolish oligomerization (Pinto & Lobe, 1996; Chen *et al*, 1998; Song *et al*, 2004). Our failure to find point mutations within the Q domain similarly points to a robustness of the Gro oligomerization interface. The deletion in *MB5* removes four evolutionarily conserved amino acids that lie outside helix AH1 (Fig 2B) that might act as an interface for recognition by other proteins. Nevertheless, it remains possible that *MB5* lethality is a consequence of reduced Gro oligomerization.

Fig 5 | Q-domain-mediated oligomerization of Gro variants. (**A**) Autoradiograph of ^{35}S-Met-labelled Gro mutant proteins run on SDS–polyacrylamide gel electrophoresis. MB12 migrates slightly faster than wild type (+), which is consistent with an amino-terminal truncation. MB5 and L36P,L87P variants run similar to wild type. (**B**) Autoradiograph of the translation reactions shown in (**A**) run on BN-PAGE. MB12 and L36P,L87P do not form a tetrameric complex. (**C**) Western blot of wild type and *MB5* extracts run on BN-PAGE. MB5 forms a high-molecular-weight, tetrameric band. (**D,E**) Pull-down analysis of ^{35}S-Met-labelled full-length, translated Gro[+] and (**D**) MB5 or (**E**) L36P,L87P or MB12 protein on GST–wild-type and GST–MB5 Q domains. Neither MB5 nor MB12 binds to an isolated Q domain in this assay. (**F**) Pull-down analysis of translated Gro[+], MB5 and MB12 proteins on GST fusions of full-length Gro[+], MB5 and MB12 proteins, showing that immobilized, full-length MB5 retains the ability to bind to translated Gro[+]. (**G**) Disruption of oligomerization in two different Q domain mutants, MB12 and L38P,L87P, reduces binding to the WRPW motif. *MB41* is a missense mutation in the WD domain that abolishes binding to WRPW (Jennings *et al*, 2006). Control input tracks represent 10% of labelled Gro protein. BN-PAGE, blue native-polyacrylamide gel electrophoresis; Gro, Groucho; GST, glutathione-*S*-transferase.

By contrast, removal of AH1 in MB12 completely prevents oligomerization *in vitro*. Translated MB12 lacks the slow oligomeric band on BN-PAGE (Fig 5B) and does not interact with the wild-type Q domain in a GST pull-down assay (Fig 5E).

Oligomerization of MB12 is probably also abolished *in vivo*, although we could not test this directly because of the greatly reduced levels of MB12 protein in the mutant embryos. An inability to homo-oligomerize probably explains the strong phenotype of

MB12, but its residual activities in certain Gro-dependent processes indicate that Gro also has oligomerization-independent activities.

Although it is clear that the WRPW and eh1 repressor peptides bind directly to the WD domain *in vivo* and *in vitro* (Paroush *et al*, 1994; Fisher *et al*, 1996; Jimenez *et al*, 1997; Jennings *et al*, 2006), such binding is also affected by mutations that impair Gro oligomerization (Fig 5G). The MB12 and L38P,L87P variants do not oligomerize (Fig 5B,E), and show greatly reduced binding to the WRPW and eh1 peptides in the GST pull-down assay (Fig 5G; data not shown). Similarly, Song *et al* (2004) reported reduced binding of GST–Brk, GST–Hkb and GST–Hairy to L38P,L87P. Most probably, these results reflect stronger binding of dimeric GST fusions to oligomerized corepressors, although we cannot exclude the possibility that the Q domain helps to structure the WD domain in solution for optimal peptide binding. Nevertheless, the *in vivo* relevance of dimeric peptide–Gro interactions remains to be determined. bHLH proteins act as dimers, but there is no current evidence that presentation of two WRPW or eh1 peptide repressor motifs is required for Gro-mediated repression.

Gro represses transcription through multiple mechanisms

In vivo, Gro can mediate 'dominant' repression, causing the silencing of all linked enhancers to a gene (Barolo & Levine, 1997). Gro has also been described as a 'long-range' repressor that can inhibit transcriptional initiation while bound to a distant (>1 kb away) enhancer element (Barolo & Levine, 1997). These observations, together with that of Gro oligomerization through the Q domain, have fuelled the predominant 'spreading' model for Gro function, in which Gro oligomerizes along the DNA through the Q domain and thereby directs heterochromatic silencing and epigenetic changes in chromatin structure (Chen *et al*, 1998; Chen & Courey, 2000; Song *et al*, 2004).

However, Gro-dependent repression does not always cause the dominant silencing of linked enhancers within a complex *cis*-regulatory region (Nibu *et al*, 2001). Moreover, Gro-mediated repression during animal development is frequently dynamic and rapidly reversible. Striped expression of *Drosophila* segmentation genes such as *hairy* and *ftz* evolves and decays within a period of approximately 30 min (Edgar *et al*, 1986), serial production of *Drosophila* embryonic neuroblasts relies on five short pulses of E(spl)-mediated repression that occur within 4 h (Doe, 1992; Jennings *et al*, 1994; Paroush *et al*, 1994), and cyclic repression by Hes proteins during zebrafish somitogenesis has a periodicity of 20–30 min (Oates & Ho, 2002).

Although our results indicate that simple Q-domain-directed oligomerization of Gro is not obligatory for its activity *in vivo*, Song *et al* (2004) found that the effects of overexpressing Gro in wing imaginal disks depend on oligomerization. Our results can be reconciled if, as seems possible, Gro mediates repression through more than one distinct molecular mechanism, with varying requirements for an intact Q domain according to the different transcription factor complexes assembled at different promoters. Local repression might predominate in dynamic developmental contexts that make use of rapidly reversible transcriptional inhibition.

METHODS

Drosophila culture and mutagenesis used standard conditions and protocols. Germ-line clone analysis, *in situ* hybridization, immunohistochemistry and GST pull-downs were carried out as described previously (Jennings *et al*, 2006). *In vitro* protein analysis was carried out using the XCell SureLock gel system (Invitrogen; www.invitrogen.com) with appropriate pre-cast gels and buffers. Blue-native gels were run with 0.1% Coomassie Blue-G250 in the cathode buffer (Schagger & von Jagow, 1991).

Supplementary information is available at *EMBO reports* online (http://www.emboreports. org).

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