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BMP-dependent gene repression cascade in Drosophila eggshell patterning

Enrica Charbonnier [a,b,c,1], Alisa Fuchs [a,1], Lily S. Cheung [d], Mrinal Chayengia [b,c], Ville Veikkolainen [c], Janine Seyfferth [a,c], Stanislav Y. Shvartsman [d], George Pyrowolakis [a,c,*]

[a] Institute for Biology I, Faculty of Biology, Albert-Ludwigs-University of Freiburg, Germany
[b] Research Training Program GRK 1104, Albert-Ludwigs-University of Freiburg, Germany
[c] BIOSS Centre for Biological Signaling Studies, Albert-Ludwigs-University of Freiburg, Schänzlestr. 18, 79104 Freiburg, Germany
[d] Lewis-Sigler Institute for Integrative Genomics, Princeton University, Princeton, NJ 08544, USA

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ABSTRACT

Bone Morphogenetic Proteins (BMPs) signal by activating Smad transcription factors to control a number of decisions during animal development. In *Drosophila*, signaling by the BMP ligand Decapentaplegic (Dpp) involves the activity of *brinker* (*brk*) which, in most contexts, is repressed by Dpp. *Brk* encodes a transcription factor which represses BMP signaling output by antagonizing Smad-dependent target gene activation. Here, we study BMP-dependent gene regulation during *Drosophila* oogenesis by following the signal transmission from Dpp to its target *broad* (*br*), a gene with a crucial function in eggshell patterning. We identify regulatory sequences that account for expression of both *brk* and *br*, and connect these to the transcription factors of the pathway. We show that Dpp directly regulates *brk* transcription through Smad- and Schnurri (Shn)-dependent repression. Brk is epistatic to Dpp in *br* expression and activates *br* indirectly, through removal of a repressor, which is yet to be identified. Our work provides first *cis*-regulatory insights into transcriptional interpretation of BMP signaling in eggshell morphogenesis and defines a transcriptional cascade that connects Dpp to target gene regulation.

Introduction

Bone Morphogenetic Proteins (BMPs) act through an evolutionarily conserved signaling pathway to regulate numerous developmental processes (Wu and Hill, 2009). Binding of BMPs to receptors triggers activation of Smad complexes which bind DNA to regulate target genes. *Drosophila* provides an excellent model to investigate transcriptional responses to BMPs. Studies in *Drosophila* have mainly focused on two processes, the patterning of the dorsoventral axis at early embryogenesis and the patterning along the anterior-posterior axis of the larval wing imaginal disc. In both cases, the fly BMP ligand Decapentaplegic (Dpp) acts as a morphogen to generate a gradient of Smad activity. Despite drastic differences in the mechanisms that generate graded activity, the transcriptional responses to the gradient share some common features (Müller et al., 2013; O'Connor et al., 2006). In both cases, expression of Dpp-target genes involves the activity of the transcriptional repressor Brinker (Brk). In the embryo, *brk* is activated in two lateral stripes in the neuroectoderm and establishes the ventral limits of genes activated by the dorsal-to-ventral Dpp activity gradient (Ashe et al., 2000; Jaźwińska et al., 1999b). In the wing disc, the tight connection between Dpp and Brk is even more pronounced, as Brk not only represses Dpp-target genes, but is itself transcriptionally repressed by Dpp (Affolter and Basler, 2007). Repression is mediated by short DNA sequences in the regulatory regions of *brk*, the *silencer elements* (*SEs*), that bind phosphorylated Mad (pMad) and Medea (the *Drosophila* Smad proteins) along with the transcriptional repressor Schnurri (Shn) (Pyrowolakis et al., 2004).

The relative contribution of Brk in BMP-target expression differs from gene to gene (Fig. 1A). A few genes in the early embryo (for example the high BMP threshold gene *race* (Wharton et al., 2004)) are directly activated by Smad complexes and do not require Brk input, while other genes (for example *dad* and *sal* in the wing or *pnr* and *zen* in the early embryo) integrate positive and negative inputs from Smad and Brk, respectively (De Celis and Barrio, 2009; Liang et al., 2012; Rushlow et al., 2001; Weiss et al., 2010). Finally, a third group of genes (for example *omb* in the wing) are directly repressed by Brk but do not require direct input by Smad complexes (Campbell and Tomlinson, 1999; Jaźwińska

* Corresponding author at: BIOSS Centre for Biological Signaling Studies, Albert-Ludwigs-University of Freiburg, Schänzlestr. 18, 79104 Freiburg, Germany.
E-mail address: g.pyrowolakis@biologie.uni-freiburg.de (G. Pyrowolakis).
[1] Equal contribution.

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et al., 1999a; Sivasankaran et al., 2000). The latter represents the most extreme situation, whereby the role of BMP signaling is restricted to relieving Brk-mediated repression.

Here, we investigate transcriptional responses to BMP signaling during morphogenesis of *Drosophila* eggshell, a proteinaceous structure derived from the follicle cells (FC) surrounding the developing oocyte. During oogenesis, the anterior-posterior gradient of Dpp, together with the dorsal–ventral gradient of the EGF-like molecule Gurken (Grk), is crucial for the formation of anterior eggshell structures, such as the two dorsal appendages (DA) and the operculum (Fig. 1B) (Berg, 2005). Both pathways converge on a number of genes, including the key patterning gene *broad* (*br*), which specifies DA primordia and is absent from the dorso-anterior cells that form the operculum primordium (Dobens and Raftery, 2000; Dobens et al., 2000; Nilson and Schüpbach, 1999; Peri and Roth, 2000; Twombly et al., 1996). Loss of Dpp signaling results in anterior expansion of *br* expression, at the expense of the operculum cell fate (Shravage et al., 2007; Yakoby et al., 2008). *Brk*, which is repressed by Dpp signaling at this stage and is excluded from anterior FCs, is required for *br* expression and DA formation (Fig. 1B) (Chen and Schüpbach, 2006; Shravage et al., 2007).

Despite the importance of Dpp and Brk in *br* regulation, *cis*-regulatory aspects as well as the relative contributions of the two factors in *br* regulation remain unclear. Here we identify *cis*-regulatory modules (CRM) for both *brk* and *br* and use genetics and reporter gene assays to address their relation to Dpp. We show that Dpp directly represses the FC-specific CRM of *brk* in a Shn-dependent manner. We then establish that Dpp signaling defines the anterior extent of the DA-primordia by repressing the activity of a recently identified enhancer of *br*, *brLate* (*brL*), and show that this effect is not direct but entirely mediated by Brk, which is in turn required for *brL* activation. Our results suggest that Brk shapes the anterior limit of *brL* expression by de-repression: Brk restricts the activity of a *brL*-repressor to anterior most FCs. We discuss our findings in the context of BMP-signaling interpretation in other developmental systems.

Materials and methods

Fly stocks and clonal analysis

Following fly lines and chromosomes were used: *brkX47*, *ywhsflp*^{12}, *UAS-dad*, *UAS-TkvQD*, *UAS-mad-RNAi* (VDRC line 12635), *FRT42BshnTD5*, *brk*^{M68}*FRT19*, *mad*^{12}*FRT40A*, *tkv*^{strII}*FRT40*, *FRT82Bmirr*$^{df-1}$ and corresponding *FRT* chromosomes carrying *ubiGFP* constructs. *mirr*$^{df-1}$ is a ~27 kb deletion removing the complete *mirr* gene and was generated by FRT/FLP-mediated recombination of the FRT-containing transposable elements d07857 and f03107 (Parks et al., 2004). *mad/brk* double mutant clones were generated using [*brk*^{BAC}]*ubiGFPFRT40A* (gift from K. Basler) in a *brk* mutant background; [*brk*^{BAC}] is a genomic rescue construct of *brk* inserted on 2L22A (Schwank et al., 2011). Experimental conditions for the generation of mosaics have been described elsewhere (Cheung et al., 2013; Fuchs et al., 2012).

Identification of silencer elements

Genomic sequences of *brk* and *br* were screened for the existence of *SEs* using GenePalette (Rebeiz and Posakony, 2004) and standard DNA sequence analysis software. We searched for matches to the originally described, 16 nucleotide long *SE*, GRCGNC(N)$_5$GTCTG (Pyrowolakis et al., 2004) and a recently described, more flexible version of the motif, GNCGNC(N)$_5$GNCTN (Gafner et al., 2013). This search identifies a total of 13 occurrences in the *brk* locus, which, with the exception of *SE*9 and *SE*12, match the more stringent consensus (see Fig. 1C and Gafner et al., 2013 for details), and a single occurrence in *brL* (see Fig. 3). In addition, we took into account *SE* variants which deviate from these two motifs, yet have been demonstrated to be functional in biochemical and/or *in vivo* reporter assays. The motifs included the *SE*-variant GRCKNC(N)$_5$GTCTG derived from the analysis of the BMP-dependent Xvent2 promoter (Yao et al., 2006), and low affinity binding sites recently identified in *brk*, *msh* (also known as *Drop*) and *reaper* (*rpr*) (Beira et al., 2014; Esteves et al., 2014; Gafner et al., 2013). The later motifs deviate from the *SE* at nucleotide positions 1 or 3 of the original SE motif. Inclusion of all these variants in the analysis of *brL* did not reveal any additional *SE*-candidates.

Reporter constructs and D transgenesis

Genomic fragments of *br* and *brk* were PCR amplified and inserted into pnlacZattB (gift of Konrad Basler), pnlacZattB-GW (Fuchs et al., 2012), pH-Stinger (Barolo et al., 2000) or pEGFPnucattB. The later was constructed by replacing the UAS-hsp70 cassette of pUASattB with a fragment from pH-Stinger (Barolo et al., 2000) that includes the multicloning site, the hsp70 minimal promoter and a nuclear EGFP reporter. Oligonucleotide primers used for the generation of the constructs were as follows (restriction site or gateway cassettes are underlined, in some indicated occasions endogenously present restriction sites were used for cloning): *brkA*: brkA_fw: CGTTCTAGCAGGGGTCACACTGTTGGCGC (endogenous XbaI site used for cloning) and brkA_rev: ttctGGTACCTGTGCCCACTGTACGTGTGACTGTGAG; *brkG1*: brkG1_fw: TTTTAGATCTACCCCCTCCTGTGTACTTCAATGC and brkG1_rev: TTTT GGTACCATGGATCCATATCCGGTAGCTGGC; *brkG2*: brkG2_fw: TTTTAGATCTAAGCTTCACT CACAGTCACACGTACAGTGG and brkG2_rev: TTTTGGTACCAGTCCATAGATCAGTTGGTG ATCGTG; *brkG3*: brkG3_fw: TGCGATGTCCCCAGCTGAATCACC (endogenous BamHI used for cloning) and brkG3_rev: TTTTGGTACCGGCTCCACTGTAGTTTTAGGTCTCC; *brkB*: brkB_fw: ttctGCTAGCGACACGATCACCAACTGATCTATGGACTTC and brkB_rev: ttctGGTACCCCTTG CGATTGCCACTGTGCGGCTCTC; *brkC*: brkC_fw: ttctTCTAGACTCTGGCTAGCTCTCCCTCTCTT TTGCAG and brkC_rev: ttctGGTACCGCTAG TTAGCAGGCGTCGACGTAGGCGC; *brkG4*: brkG4_fw: TTTTAGATCTCCAAGTTGAAACGATCGTGCAGCG and brkG4_rev: TTTTGGTACC GTGCGGTATGGTAAGATGAAGTGG; *brkD*: brkD_fw: ttctGGTACCCGATTCCGATTGTGGAT GCCACTACATAC and brkD_rev: ATACGTTCTAGACTGCCTCGCCTCGGCCG (endogenous XbaI site used for cloning); *brkG5*: brkG5_fw: TTTTAGATCTCCACTTCATCTTACCATACCGCAC and brkG5_rev: TTTTGGTACCGCGTTCTAGTTCGAAGATACGTTC; *brkG6*: brkG6_fw: GAGGACATC ATCCGTCAACCGAC (endogenous AvrII site used for cloning) and brkG6_rev: TTTT GGCGCGCCAGCCTCCGGAAATTGGTCAAGCTC; *brkG7*: brkG7_fw: TTTTCCTAGGCTCC TCGTGTAGATCAATGCCGTG and brkG7_rev: TTTTGGCGCGCCTGGCCGAATTATCGACACCT GTGC; *brL*: brL_fw: ggggACAAGTTTGTACAAAAAAGCAGGCTtcTTTCCTTTTTGCCTGGCGTC and brL_rev: ggggACCACTTTGTACAAGAAAGCTGGGTcTGTGTTGGATACTGCTGTGG, *brS*: brS_fw: ggggACAAGTTTGTACAAAAAAGCAGGCTtcGTTTTCTGCCTTCTGCCTTC and brS_rev: same as brL_rev. Mutagenesis was performed by PCR-based methods to either completely delete 16 bp long potential *SEs* or to introduce point mutations. Reporter constructs were verified by sequencing. With the exception of *brkB*14 which was cloned into pH-Stinger and inserted in the fly genome by P-element transgenesis, all other constructs were inserted by PhiC31/attB-mediated integration into chromosomal position 68A4 (Groth, 2004) or 22A3 (Venken et al., 2006).

Immunostaining and microscopy

Immunostaining of ovaries was performed as described elsewhere (Cheung et al., 2013; Fuchs et al., 2012). Antibodies included mouse anti-Br core (1:100, DSHB, The university of Iowy), rabbit anti-ßGal (1:500, Cappel), chick anti-GFP (1:500, Abcam) and Alexa-Fluor conjugated secondary antibodies (1:500, Molecular Probes). Images were acquired using a Zeiss 510 confocal microscope. Images were processed with ZEN (Zeiss, 2010) and Photoshop (Adobe Systems). Unless otherwise stated, single confocal slices at, or near the surface of stage 10 egg chambers are shown.