Roadkill attenuates Hedgehog responses through degradation of Cubitus interruptus

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The final step in Hedgehog (Hh) signal transduction is post-translational regulation of the transcription factor, Cubitus interruptus (Ci). Ci resides in the cytoplasm in a latent form, where Hh regulates its processing into a transcriptional repressor or its nuclear access as a transcriptional activator. Levels of latent Ci are controlled by degradation, with different pathways activated in response to different levels of Hh. Here, we describe the roadkill (rdx) gene, which is expressed in response to Hh. The Rdx protein belongs to a conserved family of proteins that serve as substrate adaptors for Cullin3-mediated ubiquitylation. Overexpression of rdx to different levels of Hh. Here, we describe the roadkill (rdx) gene, which is expressed in response to Hh. The Rdx protein belongs to a conserved family of proteins that serve as substrate adaptors for Cullin3-mediated ubiquitylation. Overexpression of rdx reduced Ci levels and decreased both transcriptional activation and repression mediated by Ci. Loss of rdx allowed excessive accumulation of Ci. rdx manipulation in the eye revealed a novel role for Hh in the organization and survival of pigment and cone cells. These studies identify rdx as a limiting factor in a feedback loop that attenuates Hh responses through reducing levels of Ci. The existence of human orthologs for Rdx raises the possibility that this novel feedback loop also modulates Hh responses in humans.

KEYWORDS: Hedgehog signaling, Gli, E3 ubiquitin ligase, Cubitus interruptus, Roadkill, Drosophila

INTRODUCTION

Hedgehog (Hh) signaling is a phylogenetically conserved pathway that is crucial to the growth and development of metazoans (Hooper and Scott, 2005; Lum and Beachy, 2004). Misregulation in the Hh pathway results in numerous pathologies ranging from birth defects to cancers (Beachy et al., 2004; McMahon et al., 2003; Ruiz i Altaba et al., 2002). Hh itself is a secreted protein that acts over a short range, up to about 20 cell diameters. It often acts as a morphogen (Heemskerk and DiNardo, 1994; Jessell, 2000), where differences in Hh levels across a field of cells generate distinct developmental outputs.

Hh signaling is a tightly regulated cascade that acts through Gli-family transcription factors to alter gene expression and reprogram cell fate (Hooper and Scott, 2005; Lum and Beachy, 2004). In Drosophila, Cubitus interruptus (Ci) is the only Gli-family member, and its regulation by Hh is post-transcriptional. Ci can assume three forms: the latent form Ci155, the transcriptional repressor CiR and the transcriptional activator CiA. Different levels of Hh generate different ratios and/or levels of CiR and CiA. Because they vary in their responses to CiR and/or CiA (Muller and Basler, 2000), various target genes exhibit different thresholds for activation by Hh. Thus, orchestration of CiR and CiA is crucial to the action of Hh as a morphogen.

Distinct pathways mediate the transformation of Ci155 into CiR and CiA, and these pathways respond to different levels of Hh. The latent form Ci155 is anchored in the cytoplasm by regulatory complexes that include the divergent kinesin Costal2, the protein kinase Fused and the novel protein Suppressor of fused [Su(fu)] (Sisson et al., 1997; Stegman et al., 2000). In the absence of Hh, the complex facilitates processing of Ci155 to CiR. Ci155 is phosphorylated by cAMP-dependent Protein Kinase A (PKA), Glycogen Synthase Kinase 3 (GSK3) and Casein Kinase 1 (CK1), then ubiquitylated by a Cullin1-based E3 ubiquitin ligase, and cleaved to remove regulatory and transcriptional activation domains (Dai et al., 2003; Jia et al., 2002; Jiang and Struhl, 1998; Price and Kalderon, 2002). The resulting CiR, freed from the regulatory complex, translocates to the nucleus where it represses transcription of Hh target genes (Aza-Blanc et al., 1997; Dominguez et al., 1996). Ci155 is no longer processed to CiR, and, instead, accumulates in the cytoplasm. Prolonged and/or elevated stimulation by Hh releases a second activity of Smo (Hooper, 2003), which prompts Fused kinase to inhibit Su(fu). A biochemically uncharacterized CiA is released from the regulatory complex and begins to activate transcription, while levels of Ci155 drop (Ohlmeyer and Kalderon, 1998).

The level of Ci155 is important for correct responses to Hh; overexpression of Ci155 can de-repress Hh target genes like decapentaplegic (dpp) in the absence of Hh (Dominguez et al., 1996; Hekker et al., 1997). At least three pathways, in addition to processing to Ci75, affect Ci155 turnover. In very low Hh, the novel protein Debra shunts phospho-Ci155 to the lysosome for processing to Ci75, affect Ci155 turnover. In very low Hh, the novel protein Debra shunts phospho-Ci155 to the lysosome for degradation (Dai et al., 2003). Without Debra, Ci155 accumulates and there is increased expression of its targets dpp and ptc. When Hh is high, Ci155 is no longer phosphorylated and other pathways come into play. In the eye, Cullin3 (Cul3) mediates depletion of unphosphorylated Ci155 in the presence of Hh (Ou et al., 2002; Mistry et al., 2004). Removing cul3 kills cells, so it is unknown whether excess Ci155 has consequences when Hh is high and target genes are already de-repressed. The Hect-domain protein Hyperplastic discs contributes to Ci155 turnover, but whether this is regulated by Hh and which (if any) of its pleiotropic effects are via Ci155 remains unclear (Lee et al., 2002). Thus, Hh controls levels of Ci155 via multiple mechanisms. What remains unclear is how degradation of Ci155 is triggered by high Hh and whether this downregulation is necessary for appropriate responses.

Here, we describe identification and characterization of the roadkill (rdx) gene. It is expressed in response to high levels of Hh and then downregulates Hh responses by lowering levels of Ci155. rdx-mediated attenuation of Hh signaling is essential for eye
morphogenesis. There, rdx highlights a novel role for Hh in packing ommatidia into the hexagonal array. The Rdx protein belongs to a family of substrate-specific adaptors for Cu3-based E3 ubiquitin ligase, and associates with Ci155 in vivo. Thus, Rdx identifies a negative-feedback loop through which Hh limits its own responses by targeting Ci155 for degradation. These observations raise the possibility that vertebrate orthologs of Rdx may modulate activity of the Hh pathway through regulated degradation of Gli-family transcription factors.

MATERIALS AND METHODS

Fly strains and generation of clones

Flies were cultured at 25°C under standard conditions, except where noted: rdx1 from Matthew Scott (Stanford University); rdx2 and rdx4 from T. Kornberg (UCSF); rdx2 and rdx4 from Mark Mortin (NIH); dppBS3.0: LacZ from R. Blackman (U. Indiana); UPS: Psec from R. Johnson (Johnson et al., 1995); UAS: Hh from P. Ingham (Ingham and Fietz, 1995); UAS: Ci5M from D. Kalderon (Price and Kalderon, 1999); MS1096 from K. Basler (Dominguez et al., 1996); Lz: Gal4 from U. Banerjee (Flores et al., 1998); and act5c: cry<Gal4, UasGFP [Ito et al., 1997]. UAS: Smo is described elsewhere (Hooper, 2003). All other stocks were obtained from the Bloomington Stock Center.

Clones in imaginal discs were induced using the FLP-FRT system (Xu and Rubin, 1993). Germline clones were generated using the FLP-FRT ovoD system (Chou and Perrimon, 1996). Transgene expression used the Gal4-Uas system (Brand and Perrimon, 1993).

Genotypes

yw FLP122; FRT 82B, ovoD-3R2/FRT 82B, rdx4, red, e (Fig. 3); w MS1096; UasHh[M1]brd2 (Fig. 4C); w MS1096; UasPsc[brd2] (Fig. 4D); w MS1096; eyGal4, UAS-FLP-JD1/FRT 82B, UbiGFP-3R/FRT 82B, rdx3, red, e (Fig. 4F); y w FLP122; FRT 82B, UbiGFP-3R/FRT 82B, rdx4, red, e (Fig. 4F); w MS1096; FRT 82B, w*90E, l(3)cl-R3/FRT 82B, rdx3, red, e (Fig. 4G); w MS1096; UasRdxA[58]/FRT 82B, rdx4, red, e (Fig. 4G); w MS1096; UasRdxA[58]/FRT 82B, rdx4, red, e (Fig. 4I); y w FLP122; FRT 82B, UbiGFP-3R/FRT 82B, rdx3, red, e (Fig. 5D); y w FLP122; FRT 82B, UbiGFP-3R/FRT 82B, rdx3, red, e (Fig. 5E); y w FLP122; act5c: cry'<Gal4, UasGFP+/+; UasRdxA[58]/+ (Fig. 5F); hsGAL4/uasMycRdxA[30] (Fig. 5G); y w: FLP122; FRT 82B, w*90E, l(3)cl-R3/FRT 82B, rdx3, red, e (Fig. 6A); y w: FLP122; FRT 82B, w*90E, l(3)cl-R3/FRT 82B, rdx3, red, e (Fig. 6B).

Tissue preparation and analysis

Cuticles were prepared and mounted as described previously (Struhl, 1989). Hatch rates were determined 48 hours after 4-hour egg collections by counting empty vitelline membranes (hatched) and intact eggs (unhatched). Wings were mounted in 50% Canada balsam and 50% methyl salicylate. Standard histological methods were used for thin sections of adult (unhatched). Wings were mounted in 50% Canada balsam and 50% methyl salicylate. Standard histological methods were used for thin sections of adult eyes (Bentrop et al., 1997) and for SEM (Moses et al., 1989), which was used for 1:20,000 dilution.

Mapping and sequencing

Recovered genomic sequence adjacent to the rdx1 P-element insertion (Bier et al., 1993) and used to screen a Drosophila 0-24 hour embryonic cDNA library (Clontech). A 0.8 kb EcoRI/EcoRI fragment from the 3' end of the longest cDNA was used to rescreen the library. Restriction mapping of the five recovered cDNAs revealed two splice variants corresponding to RdxE and RdxA. cDNA sequence (CU Cancer Center DNA Sequencing & Analysis Core) was compiled with Sequencher software (Gene Codes). EMS mutations were identified by PCR amplifying genomic DNA from homozygous mutants (Laird et al., 1991), followed by cloning and sequencing (Maniatis and Sambrook, 1989). Putative mutations were confirmed with independent PCR products. Splicing of rdx was analyzed by sequencing RT-PCR products of mRNAs derived from homozygous mutants. Chromatograms were evaluated manually; sequence downstream of splice variations was read by scoring primary and secondary peaks. Northern and Southern blotting followed standard procedures (Maniatis and Sambrook, 1989).

Rdx-expressing transgenes were generated by inserting a BamHI-XhoI fragment containing a RdxA cDNA (EST LD08515) into the BgII-XhoI sites of pUAST (Brand and Perrimon, 1993). For MycRdxA, PCR was used to replace the 5' UTR and start codon with a Kozack sequence, a start codon and a Myc epitope. The correct sequence was confirmed and the modified RdxA cDNA was cloned into the KpnI-XhoI sites of pUAST. Transgenic flies were obtained by standard procedures (Spradling and Rubin, 1982). Transgenic activity was confirmed by rescue of rdx mutants; expression either ubiquitously or in the pattern of ptc rescued viability of rdx1, rdx2/rdx6 and rdx2/rdx6, but not rdx2/rdx6.

RESULTS AND DISCUSSION

rdx gene structure

The rdx locus was identified by an enhancer trap with embryonic expression in a pattern suggesting Hh-regulation (a gift from M. Scott, Stanford University). When genomic DNA flanking the insertion was used to screen a Drosophila embryonic cDNA library, we obtained cDNAs that initiated near the enhancer trap insertion and spliced into a cluster of seven downstream exons (rdxE in Fig. 1A). These cDNAs represented the predicted gene CGI01235 spliced into the predicted gene CGI9924 (Berkeley Drosophila Genome Project, Release 3.0). ESTs recovered by the BDGP identified four additional isoforms (CG9924-A-D), which differ in their 5' ends but which share the cluster of seven downstream exons with rdxE, and are designated rdx1-rdx6 (Fig. 1A).

We used northern blots (not shown) to confirm the presence of transcripts corresponding to rdxA and rdxE in embryos. A probe unique to rdxE (exon 5) detected transcripts of ~4 kb, while probe specific to rdxA (exon 6) detected the cluster of transcripts ~2.1-2.5 kb. A probe derived from the cluster of downstream exons detected

Hoechst 33258 at 1 µg/ml in PBS for in 4 minutes, rinsed and mounted in 1:1 glycerol:PBS. Confocal images were captured with Zeiss Pascal LSM and manipulated with Adobe Photoshop.

In situ hybridization was carried out using digoxigenin-labeled, antisense riboprobes, according to standard procedures (Jiang et al., 1991; Tautz and Pfeifle, 1989). rdxA-specific probe used a 180 bp EcoRI fragment from exon 6. rdxE probe used an EcoRI EcoRI fragment derived from exon 5. Exon 11-13 probe used a PCR product from cDNA #5-2. Fragments subcloned into Bluescript (Stratagene) were used for in vitro transcription.

Wing imaginal discs were prepared for western blotting as described by Ohlmeyer (Ohlmeyer and Kalderon, 1998). For immunoprecipitation, embryos were collected overnight, heat shocked at 38°C for 1 hour, recovered at 25°C for 4 hours, and then lysed and analyzed as described previously (Ogden et al., 2003). Myc (9E10) and control immunoprecipitates (FlagM2, Sigma) each used 1 µg purified IgG. Ci was detected with affinity purified rabbit 3.3 antibody at 1:5000 (P. Ingham). α-tubulin antibody (Sigma) was used at 1:20,000 dilution.
Fig. 1. rdx gene structure. (A) The rdx genomic locus spans 64 kb in cytological band 88A4. Exons are represented by blue boxes and numbered underneath; lines indicate splices; light blue indicates start codon; red indicates stop codon; arrows indicate direction of transcription (towards the centromere). Four alternative transcriptional start sites generate five transcripts (forms A-D) that share exons 7-13. Exons 1 and 2 are unique to the B form. Exons 3 and 4 are unique to the C/D forms. C differs from D by lacking seven nucleotides in the 5′ UTR at the end of exon 3. Exon 5 is unique to the E form (represented by our cDNA 5-2; predicted to be the nested gene CG12537). Exon 6 is unique to the A form. (B) Mutants are mapped onto the gene structure. Exons are indicated by boxes, where grey indicates UTRs, red indicates coding sequence unique to each form, green indicates coding sequence for the conserved MATH domain and magenta indicates coding sequence for the conserved BTB domain. The numbers above each indicates the amino acid coordinates. Unique sequences are in red; the MATH domain is in green; the BTB domain is in blue. The asterisk identifies alternative termination for 3′ UTRs. rdx-A is a LacI::G4 insertion 33 nt upstream of the start of exon 5. rdx-A is a complex rearrangement of the region upstream of exon 5, generated by imprecise excision of rdx-A. Black arrowheads indicate the missense mutations in rdxB (G to T in exon 5 encoding Q193H and A to T in exon 10 encoding D653V), and the splice junction mutation in rdxA (an A to G change in the 23rd nt of the intron between exons 12 and 13). (C) Block diagrams indicating the four proteins encoded by the rdx locus. The numbers above each indicates the amino acid coordinates. Unique sequences are in red; the MATH domain is in green; the BTB domain is in magenta. The arrow indicates the convergence of amino acid sequences.

Fig. 2. rdx expression in embryos. rdx mRNA was detected with probe to exons shared by all transcripts (A-I). Probes specific to rdxA or rdxE detected similar expression patterns (not shown). Lateral views show: (A) stage 1-2; (B) stage 4 with pole cells (pc); (C) stage 5; (D) stage 6; (E) stage 8 with anterior midgut (am) and posterior midgut (pm); (F) stage 10; (G-I) stage 11 with stomodeum (sto), ninth abdominal segment (A9), CNS (n) and mesoderm (ms). A ventral view of stage 10 is focused under the ectoderm to show neuroblasts in F. (H) Parasagittal optical section of an embryo homozygous for cC shows loss of rdx expression in the ectoderm and mesoderm, but retention in the head, in A9 ectoderm, and in the CNS. A similar view of a ptc2 homozygous embryo (I) shows extensive ectopic expression of rdx in the ectoderm and mesoderm. The gaps in rdx expression in the ectoderm correspond to posterior compartment cells expressing En (not shown). (J,K) At stage 10/11, En expression (blue) is nestled between stripes of rdx expression (brown), as detected by β-Gal in rdx- embryos. (L) Stage 12 with salivary gland primordium (sg). Dorsal is upwards (except in F) and anterior is towards the left in all panels.

rdx expression is regulated by Hh

rdxA, rdxE and the initial enhancer trap produced expression patterns that were indistinguishable from those of a probe common to all rdx forms (Fig. 2; data not shown). Maternally deposited rdx transcripts were detected in early embryos (Fig. 2A), but disappeared during mid-cleavage stages (not shown). The first zygotic transcripts appeared in pole cells (Fig. 2B). During cellularization of the blastoderm, rdx transcripts appeared in two broad stripes in the head, in seven narrower stripes along the segment primordium, and in a ring surrounding the pole cells (Fig. 2C). Seven additional stripes appeared during germ band extension (Fig. 2D), so that by stage 8, rdx was expressed in 14 evenly spaced ectodermal stripes characteristic of segment polarity genes (Fig. 2E). At this time, strong expression was seen in the anterior and posterior midgut primordia. During stage 9/10, expression appeared in a subset of neuroblasts (Fig. 2F). During stage 10 each segmental stripe split so that by stage 11, ectodermal expression consisted of two thin stripes corresponding to the anterior and posterior margins of the former
stripe (Fig. 2G). At this time, strong expression was seen in the mesoderm (not shown). As germ band retraction began, expression faded from most of the ectoderm, but was retained in the salivary glands and in abdominal segment 9 (Fig. 2L). After stage 14, rdx expression was detected only in the clypeolabrum, anal plate and salivary glands (not shown).

The double stripes of rdx expression during germ band extension are reminiscent of those of patched (ptc), the canonical Hh responsive gene. Using Engrailed (En) to mark the Hh-expressing cells, we found that the segmentally repeated stripes of rdx were centered around Hh during stage 8/9 (not shown), while the double stripes of rdx during stage 10/11 flanked cells expressing Hh (Fig. 2J,K). Thus, rdx is expressed in cells known to be responding to Hh. To test whether expression of rdx requires Hh signaling, we examined rdx expression in ciCe mutant embryos. This allele produces a truncated Ci protein that mimics CiR and constitutively produces Hh target genes (Methot and Basler, 1999). rdx expression in ciCe embryos was lost in the segmented ectoderm by stage 11, though Hh-independent expression remained in neuroblasts and in the ectoderm of A9 (Fig. 2H). To test whether Hh signaling is sufficient to activate rdx expression, we used ptc mutant embryos to activate Hh signaling throughout the anterior compartment (Ingham et al., 1991). In ptc mutants, rdx expression filled the anterior compartment in the ectoderm, and was also extensively activated in the mesoderm (Fig. 2I). Thus, rdx is a Hh target gene; it is normally expressed in cells adjacent to those expressing Hh, and Hh signaling is both necessary and sufficient to activate rdx expression.

**rdx alleles**

Five additional alleles of rdx were identified by failure to complement the enhancer trap allele rdx1. The molecular lesions are mapped onto rdxE in Fig. 1B. A LacW insertion near the start of exon 5 is responsible for rdx1, as precise excision of the P-element restored viability (not shown). rdx2 and rdx3 (gift of T. Kornberg) are also LacW insertions with lethality and β-galactosidase (β-gal) expression patterns similar to rdx1. By Southern blotting, their insertions are within 100 nucleotides of the start of exon 5 (not shown). rdx4 is a complex rearrangement (not shown) that was generated by imprecise excision of rdx1. rdx5 and rdx6 derived from an EMS mutagenesis (Mortin et al., 1992). rdx6 contains a point mutation in the splice donor between exon 12 and exon 13. Using RT-PCR, this allele generated mRNAs that retain intron 12/13, as well as wild-type mRNAs. Translation into intron 12/13 would truncate Rdx at the end of the BTB domain. rdx6 included two missense mutations: Q193H in sequences unique to RdxE, and D653V in a highly conserved residue of the BTB domain. X-ray structure of a BTB domain predicts that the side chain of D653 hydrogen bonds with the peptide backbone (Ahmad et al., 1998), and disruption of this hydrogen bond could destabilize the BTB domain.

All six rdx alleles, in all combinations, caused recessive lethality. rdx1 or rdx6 homozygotes hatched, but the sluggish, slow-growing larvae seldom survived beyond the first instar. Fewer rdx5 larvae hatched, and none survived beyond first instar. rdx6 embryos seldom hatched. Homozygotes for Df(3R)red1, a deficiency that includes many genes in addition to rdx, never hatched. These data defined the allelic series Df(3R)red1>rdx5>rdx6=rdx1> wild type. This is consistent with the molecular lesions: rdx1 and rdx5 should affect only the E form and thus be partial loss of function; rdx6 should affect all forms but make both wild-type and mutant product; and rdx4 should disrupt the protein structure for all forms. In the rest of this work, we use rdx6 to define rdx loss-of-function phenotypes.

**A maternal effect for rdx**

As Hh regulates rdx transcription during segmentation, we expected a role for rdx in Hh signaling or segmental patterning. However, segmentation, as assessed by cuticle pattern, was normal in rdx mutant embryos (not shown). To ask whether maternally deposited rdx mRNA might mask a role for rdx in early patterning, we used germline clones (Chou and Perrimon, 1996) to generate embryos lacking both maternal and zygotic rdx. GLC of the weak loss-of-function alleles rdx4 or rdx5 yielded embryos that developed and hatched normally. No eggs were recovered from rdx3 GLC, suggesting that rdx is essential for oogenesis. Embryos derived from rdx6 GLC displayed a spectrum of developmental defects ranging from normal patterning (2%), through a complete failure of cellularization (75%). An intermediate class (23%) included errors in segmental patterning. It also included embryos that developed at one end, but that slumped at the other end into an

**Fig. 3. rdx is necessary for the meiotic to mitotic transition.** Cuticle from a wild type Canton-S embryo (A) shows evenly spaced denticle bands on the ventral surface, telson structures at the posterior end (t) and internal mouthparts at the anterior end (m). (B) Hoechst staining of DNA visualizes mitoses in a wild type Canton-S blastoderm stage embryo. The cuticle of a rdx6 GLC embryo (C) shows intact telson (t), a fusion of A2–A8, intact T3 and A1 denticle bands, and a large anterior hole extruding malformed mouthparts (m). In wild type (B), nuclei are uniformly spaced, synchronized divisions occur, and chromatin is distributed evenly between daughter nuclei. In a rdx6 GLC embryo (D), mitoses are asynchronous, nuclear spacing is uneven, mitotic figures are highly abnormal (inset) and much of the DNA has sunk into the interior of the embryo (internal staining is out of focus).
amorphous mass, a phenotype most accurately described as roadkill (Fig. 3C). The failure of cellularization in most rd5 CLC embryos reflected severe defects during the early mitotic cycles (Fig. 3D). Abnormalities included mitotic asynchrony, uneven nuclear spacing, failed anaphase resolution and multipolar mitotic figures. The requirement for rdx function in early mitoses is strictly maternal, as wild-type sperm did not rescue the defects of rd5 CLC embryos (not shown). As rdx5 or rdx6 homozygous clones were the same size as their wild-type counterparts in eye and wing imaginal discs (Figs 4 and 5), we conclude that the mitotic requirement for rdx is limited to the earliest mitoses in embryos. As there is no requirement for Hh signaling in the female germline, the requirement for rdx in the meiotic/mitotic transition must represent a Hh-independent function.

MEL-26, the C. elegans of the MATH/BTB protein family, promotes the meiotic/mitotic transition through degradation of the MEI-1/kaatinan (Mains et al., 1990; Pintard et al., 2003; Xu et al., 2003). Maternal loss of MEL-26 leads to a mitotic catastrophe similar to that observed for rdx. This suggests that a phylogenetically conserved function for the Rdx/MEL-26 family may be to assist in the meiotic/mitotic transition by targeting Katanin/MEI-1 for degradation.

A role for rdx in Ci regulation

As the mitotic failure precluded studies of rdx during embryonic segmentation, we turned to imaginal discs to investigate the connection between rdx and Hh. In the developing wing, Hh is made in the posterior compartment while anterior compartment cells respond to Hh because they express Ptc and Ci (schematized in Fig. 4N). Deep in the anterior compartment, cells see no Hh; Ci155 is processed to CiR, resulting in low levels of Ci155 and repression of target genes. Immediately adjacent to the compartment border, high levels of Hh activate Ci and block its processing to CiR; target genes with the highest threshold for Hh are expressed, and levels of Ci155 are low. In between are moderate levels of Hh; Ci155 accumulates but is not activated, CiR may be reduced and target genes with lower thresholds for Hh are induced.

rdx expression was detected in a strip of cells just anterior to the AP border (Fig. 4A,B). Its posterior edge coincided with that of Ci155, at the compartment border. It extended slightly anterior to the En expression that marks the highest responses to Hh, and overlapped almost exactly with the decreased Ci155 immediately adjacent to the compartment border. This rdx expression is most similar to the domain where ptc is induced by Hh. Moreover, rdx expression in the wing is Hh dependent: it was expanded with overexpression of Hh and disappeared when Hh response was blocked by overexpression of Ptc (Fig. 4C,D).

In rdx loss-of-function clones, we found increased levels of Ci155, but only when those clones were within ~10 cells of the compartment border (Fig. 4F). Ci155 also accumulated in rdx6 clones near the border, though levels were somewhat lower (Fig. 4E). As Ci155 levels were only affected in cells that lost rdx function (e.g. Fig. 4F), the effect of rdx on Ci is cell-autonomous. We found no change ci transcription in clones lacking rdx (using β-gal from ct-lacZ as a reporter, not shown), indicating that the effects of rdx on Ci155 are post-transcriptional. Taken together, these data suggest that rdx acts as part of a negative feedback loop that downregulates levels of Ci155 in response to Hh.

Wings filled with unmarked rdx clones (28 of 40 wings) showed a mild elongation of the anterior compartment (Fig. 4G). However, rdx loss-of-function clones had no discernible effect on expression of the Hh target genes En, Ptc, Collier or dpp. This is consistent with previous observations that forced overexpression of Ci155 has little phenotypic consequence if it is limited to the domain immediately adjacent to the compartment border (Dominguez et al., 1996; Hepker et al., 1997; Wang et al., 1999). The molecular basis for the altered wing morphology could not be determined.

To ask whether rdx could affect Ci155 beyond the range of Hh, we overexpressed RdxA in the wing pouch (red in Fig. 4I). This reduced Ci155 levels both at the compartment border and deep in the anterior compartment (Fig. 4L). Ptc and En induction was reduced or blocked (Fig. 4J,K), while dpp expression broadened (Fig. 4K).

The adult wings were smaller than normal, with ectopic veins anterior to vein 3 (Fig. 4I). This loss of some targets and activation others is most consistent with reduction in both CiR and CiA, in all Ci activity (Methot and Basler, 1999). In western blots of wing imaginal discs, we found that overexpression of Rdx reduced levels of both Ci155 and Ci75 (Fig. 4M; the modest reduction is all that could be expected, given that Rdx was overexpressed only in the wing pouch and not in the remainder of the disc). Taken together, these data show that rdx attenuates levels of all forms of Ci in response to Hh. This pathway for Ci regulation plays a minor role in wing development but may be more significant in other tissues.

rdx regulates levels of Ci155 in the eye

In the eye imaginal disc, a wave of Hh signaling initiates differentiation as it propagates across the disc (Fig. 5A). Between undifferentiated and differentiating tissue lies the morphogenetic furrow (MF), a contraction of the columnar epithelium. In front (anterior) to the MF, CiR maintains the undifferentiated state by repressing photoreceptor specification (Heberlein et al., 1993; Hsiung and Moses, 2002; Ma et al., 1993). Behind (posterior) to the MF, differentiating photoreceptors make Hh (Lee et al., 1992). Within the MF, undifferentiated cells fall under the influence of Hh from adjacent photoreceptors; CiR production is blocked, and a new row of photoreceptors is initiated. Thus, the MF/differentiation moves from posterior to anterior across the eye imaginal disc. Hh signaling, as measured by continuing expression of ptc, remains active behind the MF (Shyamala and Bhat, 2002). A developmental role for this Hh signaling has not yet been identified.

In the eye, Hh regulates two distinct pathways that effect Ci155 turnover (Fig. 5A,C,D). In front of the MF, Ci155 is processed to CiR via phosphorylation and the Slimb/Cul1-based ubiquitin E3 ligase. Within the MF, Hh blocks that pathway and Ci155 accumulates. Behind the MF, Ci155 is depleted by a Cul3-dependent pathway that involves neither PKA nor Slimb (Ou et al., 2002). That pathway requires Hh signaling, as Ci155 accumulates in smo clones behind the MF. As Cul3 is present throughout the eye disc (Ou et al., 2002), it is not known why Cul3 depletes Ci155 only behind the MF.

rdx is expressed in all cells posterior to the MF, with highest levels in photoreceptor clusters and cone cells (Fig. 5B,C, and not shown). rdx expression in the eye is Hh dependent, as it is in wing and embryonic ectoderm; it fills the eye when Hh is overexpressed, and disappears when Ptc is overexpressed (not shown). In loss-of-function rdx5 clones, Ci155 levels increased behind (posterior to) the MF but not in front of (anterior to) the MF (Fig. 5E). This accumulation of Ci155 was cell-autonomous, limited to the cells lacking rdx. Thus, rdx, like cul3 (Ou et al., 2002), acts to reduce levels of Ci155 behind the MF but has little effect on Ci155 within or in front of the MF. To determine whether Rdx could affect Ci levels throughout the eye, we misexpressed RdxA in clones (Fig. 5F). We found that ectopic Rdx could destabilize Ci155 within and anterior to the MF. Thus, Rdx expression is both necessary and sufficient for degradation of Ci155 throughout the eye.
Fig. 4. *rdx* acts in a feedback loop to reduce levels of Ci. Wing imaginal discs are oriented with anterior towards the left and genotypes in the upper right-hand corner. (A-D) *rdx* expression is detected by β-gal (green) in the enhancer trap line *rdx*^+/+ (*rdxZ*). In wild type (A, B), *rdx* is in a strip of approximately five cells along the compartment border. En expression (red in A) marks the posterior compartment, with additional expression extending approximately three cells into the anterior compartment. *rdx* expression overlaps the anterior edge of the En expression (inset in A). The anterior edge of Ci155 expression (red in B,E,FL) marks the compartment border. The anterior edge of *rdx* coincides with that of Ci155 (inset in B), so *rdx* expression abuts the compartment border. The Hh-dependence of *rdx* expression (green) is demonstrated by its expansion when Hh is overexpressed (C); or by its loss when Ptc overexpression suppresses Hh responses (D). *rdx*^6^ (E) and *rdx*^5^ (F) loss-of-function clones are marked by loss of GFP (green). Clones within six to ten cells of the compartment border (arrowheads near white line) accumulated Ci155 (red), while clones farther from the border (arrow) did not. Filling wings with unmarked *rdx*^5^ clones caused a slight overgrowth of the anterior compartment (G) compared with wild type (H); the arrowhead indicates the compartment border (just anterior to vein 3) while the outline of a wild type wing is superimposed for comparison. RdxA (I,K-M) or MycRdxA (J) was mis/overexpressed using the Gal4 line MS1096. The resulting expression of Rdx is demonstrated by Myc (red in J): highest in the dorsal wing pouch (large bracket), low in part of the notum (small bracket) and off in the remainder of the disc. This reduced levels of Ci155 (red in L) and prevented Hh-dependent En induction (blue in L) in the anterior compartment (marked by the edge of Ci155 expression). It reduced levels of Ptc (green in J) near the compartment border, but broadened expression of *dpplacZ* (K). The resulting wings (I) had small anterior compartments and ectopic veins anterior to vein 3. Western blots (10 wing discs/lane) demonstrate a modest reduction (approximately halved) in both Ci155 and Ci75 when Rdx expression is driven by MS1096 (M). (N-P) Levels of Hh, Ptc, *rdx*, Ci155, CiR across the anteroposterior axis (x-axis) of wing imaginal discs, with levels indicated on the y-axis. The vertical broken lines indicate the border between the anterior (Ant) and posterior (Post) compartments. In wild type (N), *rdx* is expressed adjacent to the compartment border, where Hh activates Ci; there it reduces Ci155. With *rdx* loss (O), Ci155 is elevated adjacent to the compartment border, though this has little effect on Hh target gene expression. Ectopic Rdx (P) reduces both Ci155 and CiR, resulting in dysregulation of Hh target gene expression.
A schematic (A) shows Ci155 levels (red) across the disc, with the Hh-secreting photoreceptors posterior to the MF in grey. Anterior to the MF, in the absence of Hh, Ci155 is processed via Cul1 (blue arrow) to CiR. Posterior to the MF, Ci155 is degraded (red blobs) via CuI3 (green arrow). (B) rdx as detected by β-gal activity (blue) in rdxZ/+ discs (rdxZ), is found posterior to the MF, in the ocellar region, and at the compartment border of the antennal portion (arrow). At higher resolution (C), nuclear-localized β-gal protein in rdxZ (green) co-localized with Elav (not shown) in all photoreceptors and cone cells, while Ci155 (red) accumulated in the intervening cells. β-Gal protein is also found in many basal nuclei, corresponding to the position of lattice cell nuclei (not shown).

(D, E) rdx clones are marked by loss of GFP (green), while Ci155 is in red. The three arrows (left to right) indicate, respectively, clones behind, within and in front of the MF. The hypomorphic allele rdx1 was used as a control to illustrate normal patterning (D). In clones of the loss-of-function allele rdxZ (E), Ci155 accumulated behind but not in front of the MF: Rdx overexpression (F, marked by GFP in green) reduced levels of Ci155 (left panel, red in right panel) within or just in front of the MF (arrow). (G) When control hsGal4 embryos (+) or hsGal4/MycRdxA embryos (+R) were immunoprecipitated for Myc (IP Myc), Ci155 but not Ci75 was detected in the immunoprecipitates. Neither Ci155 nor Ci75 were detected in control immunoprecipitates using Flag antibody (IP control). Lyssates (1% and 0.2% loaded) give estimates of sensitivity. (H) The relationship of Hh, Rdx, Cul3 and Ci. Hh induces Ci155 to activate transcription of rdx (curved red arrow). Then Rdx acts with Cul3 (blue curved arrow) to degrade Ci155 (red blobs). The resulting reduction in Ci155 then attenuates Hh responses (red outlined arrow).

The similar effects of rdx and cul3 on Ci155 raise the possibility that Rdx and Cul3 act in the same pathway. Indeed, its paired MATH and BTB domains suggest that Rdx acts as a substrate-specific adaptor, bringing Ci155 to a Cul3-based ubiquitin E3 ligase. In this scenario, ubiquitously expressed Cul3 depletes Ci155 only behind the MF, because that is where rdx is expressed; when Rdx is ectopically expressed, Ci155 is ectopically degraded. If Rdx acts as an adaptor bringing Ci to Cul3, then a small fraction of Ci should be associated with Rdx in cells. To test for association of Rdx and Ci, we overexpressed Myc-tagged Rdx in embryos, and then probed Myc immunoprecipitates for Ci (Fig. 5G). We found a small fraction of Ci155 (significantly greater than 0.2%) in MycRdx immunoprecipitates, while Ci75 was not detected (threshold of detection, ~0.2%). We conclude that in vivo, Rdx binds Ci155 better than Ci75. Taken together, these data suggest that Rdx regulates degradation of Ci155 by acting as a specificity factor bringing Ci155 to Cul3. However, we cannot eliminate the possibility that Rdx may act through a Cul3-independent mechanism.

rdx reveals a role for Hh in the eye after photoreceptor recruitment

What might be the role of Hh-dependent depletion of Ci155 in the eye? Small rdx clones lacked ommatidial bristles, but produced adult eyes with otherwise normal external morphology (not shown). In eyes that mostly comprised rdx mutant cells, the ommatidia were uneven in size, misaligned and often lacked or duplicated ommatidial bristles (Fig. 6B). MF movement, photoreceptor and cone cell specification (monitored by morphology, Senseless and Elav expression) were normal in these rdx mutant eyes (not shown). Sections through mutant ommatidia revealed the correct number of photoreceptors, but the photoreceptor clusters were loose, imperfectly aligned and irregularly spaced (Fig. 6B). Thus, rdx has a role in retinal patterning after specification of photoreceptor cells and during the rectification of the ommatidial lattice.

To investigate the cellular basis for the rdx phenotype, we examined retinas at 36–42 hours of pupation, when the non-neural cells (cone, pigment and bristle cells) are moving into their final pattern. The spatial relationships of these non-neural cells are shown in Fig. 6D. rdxZ pupal ommatidia (Fig. 6E) showed misaligned 1° cells (40% of ommatidia) that sometimes failed to enclose cone cells (6%), as well as reduced numbers of cone cells (4%), inappropriate numbers of lattice cells (56%), ommatidial fusions (2%) and misplaced bristles (19%). As cone and 1° cells organize lattice cells and prevent their apoptosis (Cagan and Ready, 1989; Cordero et al., 2004; Miller and Cagan, 1998; Rusconi et al., 2000; Wolff and Ready, 1991), the ommatidial fusions and lattice cell defects are likely to be secondary to the problems with cone and 1° cells. We conclude that loss of rdx function interferes with patterning in the pupil retina, probably at the level of the cone and 1° cells.

The dramatic effect of rdx on Ci155 levels suggested that rdx is acting in retinal patterning via Ci and the Hh pathway. Indeed, hh– or smo– clones generate disorganized ommatidia similar to those in
Fig. 6. *rdx* regulates ommatidial packing. Eyes comprised of mostly *rdx* mutant cells were generated by mitotic recombination, where the wild-type daughter cells were eliminated by a cell-lethal mutation (A,B,E). SEM of hypomorphic *rdx* mutant eyes (A, left and middle panels) showed wild-type patterning, including an ordered hexagonal array of ommatidia with bristles at alternate interstices; *rdx* loss-of-function eyes had disrupted ommatidial organization and loss of bristles (B, left and middle panels). Cross-sections of *rdx* mutant eyes (A, right panel) showed normal patterning; the rhabdomeres of seven photoreceptors (dark spots) per ommatidium are arranged in a distinctive trapezoidal pattern. Cross-sections of *rdx* eyes had seven rhabdomeres in most ommatidia, but their packing was looser and not so clearly trapezoidal (B, right panel). The cellular architecture of apical surface of pupal eyes ~36 hours after puparium formation is visualized with Armadillo (Arm) (C,D,E) and schematized (D,F). Wild type (C,D) shows the regularly packed hexagonal array with the appropriate arrangement of four cone cells (c), two primary pigment cells (1°) and 12 lattice cells – six secondary pigment cells (2°) along the faces of the hexagon, with bristle (b) or tertiary pigment cells (3°) at alternating vertices. The lattice cells are shared between adjacent ommatidia. The junctions between 1° cells always align perpendicular to the anteroposterior axis and always contact 2° cells. *rdx* mutant eyes (E,F) showed misaligned 1° cells (green diamonds), 1° cells failing to wrap around cone cells (yellow stars) and displaced bristles (red triangles).

Fig. 7. Hh signaling contributes to organization of the ommatidial array. (A-D) Scanning electron micrographs of the surface of adult eyes. (E-H) Arm staining used to show the cellular structure of pupal eyes, ~36-42 hours (E-G) or ~28 hours (H). Anomalous structures are indicated as follows: red stars for missing or duplicated cone cells; the double red star in H indicates a cone cell cluster with a round cell characteristic of apoptotic morphology; yellow stars indicate 1° cells failing to enclose cone cells; green diamonds indicate 1° cells intersecting with tertiary cells; red triangles indicate missing or misplaced bristles. (I-L) Schematics of a pupal ommatidium (see Fig. 6 legend for details). Dorsal is upwards and posterior is towards the left; genotypes are indicated under each panel.
rdx mutant eyes, though this has been attributed to a distortion of the MF, rather than to a direct effect of Hh (Dominguez, 1999; Heberlein et al., 1993; Ma et al., 1993). Moreover, hyperactivation of Hh responses (via insufficient ptc) causes a variety of morphological defects in the differentiating eye (Thomas and Ingham, 2003). To determine whether Hh and Ci affect ommatidial organization after the MF has passed, we used Lozenge:Gal4 (Lz:G4) to drive UAS:transgene expression behind the MF (Flores et al., 1998). The effects of Hh overexpression were limited to the anterior margin, with some reduction and with mildly disordered bristles and ommatidial packing (Fig. 7B). The pupal retinas showed mild defects near one edge (Fig. 7F), with occasional lost or excess cone cells (4%), mis-oriented or incompletely wrapped 1st cells (44%) and excess lattice cells (55%). Expression of Ci5M, a Ci whose mutated PKA sites stabilize it and prevent its processing to a transcriptional repressor (Price and Kalderon, 1999), caused loss of ommatidial bristles (40-50%) and mildly disordered ommatidial packing (Fig. 7C). The corresponding pupal ommatidia (Fig. 7G) showed occasional loss of cone cells (2%), failure of the 1st cells to wrap around the equatorial cone cells (12%) and excess lattice cells (38%). As these defects were not seen with Lz:G4 alone (Fig. 7E), we conclude that excess Ci can interfere with 1st and lattice cell patterning. To maximally stimulate Hh signaling, we used a Smo transgene whose high level of expression makes it a potent activator (Hooper, 2003). Lz:G4+UAS:Smo generated rough eyes, with defects most pronounced in the posterior (Fig. 7D). At ~24 hours, pupal retinas were relatively normal (not shown), although 1st cells were often misaligned (27%). By ~30 hours, pupal ommatidia had reduced numbers of cone cells (16%) and of lattice cells (75%), with occasional cone cells adopting a rounded morphology characteristic of apoptotic cells (Fig. 7H). Apparently, Smo overexpression causes cone cell death in the pupal retina. Photoreceptor differentiation, assessed by counting rhodopsinocytes in pupal retinas, was unaffected in these experiments (not shown).

Taken together, these data show that overstimulation of the Hh pathway interferes with behavior of the non-neuronal cells in the pupal retina. These effects are subsequent to and independent of the role of Hh in the MF. The similarity of the phenotypes resulting from Hh overstimulation, from Ci overexpression and from rdx loss of function suggests that rdx affects retinal patterning via Ci. Without this fine-tuning by rdx (e.g. Fig. 5H), excess Ci generates excess Hh response, which ultimately interferes with retinal patterning.

**Summary and future prospects**

rdx encodes a protein belonging to a phylogenetically conserved protein family of substrate-specific adapters for Cullin3-based ubiquitin E3 ligases (Pintard et al., 2003; van den Heuvel, 2004; Xu et al., 2003). rdx loss-of-function and gain-of-function studies suggest that rdx has at least two substrates – a regulator of early embryonic mitoses and the Hh regulated transcription factor Ci155. Our data support a model where Rdx regulates the Hh-dependent degradation of Ci by acting as the adaptor that presents Ci to the Cull3-based E3 ubiquitin ligase. Because rdx is expressed in response to Hh, rdx is involved in a novel regulatory loop that attenuates Hh responses through reducing levels of Ci. In the wing, this feedback regulation of Ci by rdx plays a minor role, but in the eye it is essential for proper packing of ommatidia into a hexagonal array.

Hh is key regulator in human health (Beachy et al., 2004; McMahon et al., 2003; Ruiz i Altaba et al., 2002). The haploinsufficiency of Ptc in humans (Johnson et al., 1996) and its activity as a morphogen in the spinal column (Jessell, 2000) argue that the level of Hh response is often crucial. Although there are differences in the Hh pathway between flies and vertebrates, many regulatory mechanisms are conserved (Hooper and Scott, 2005; Huangfu and Anderson, 2006; Lum and Beachy, 2004). In particular, Gli2 and Gli3 are regulated much like Ci, becoming repressors or activators, depending on levels of Hh. The Rdx ortholog SPOP lies in 17q21.33, a chromosomal region that has been linked with ovarian cancer and cervical immature teratoma (Bernardini et al., 2005; Miliaris et al., 2005). Future studies will determine whether the Rdx orthologs SPOP or LOC339745 modulate Gli levels and Hh-mediated responses, and even contribute to cancer.

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**References**


