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curled Encodes the Drosophila Homolog of the Vertebrate Circadian Deadenylase Nocturnin

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ABSTRACT

Drosophila melanogaster curled, one of the first fly mutants described by T. H. Morgan >90 years ago, is the founding member of a series of curled wing phenotype mutants widely used as markers in fruit fly genetics. The expressivity of the wing phenotype is environmentally modulated, suggesting that the mutation affects the metabolic status of cells rather than a developmental control gene. However, the molecular identity of any of the curled wing marker mutant genes is still unknown. In a screen for starvation-responsive genes, we previously identified the single fly homolog of the vertebrate nocturnin genes, which encode cytoplasmic deadenylases that act in the post-transcriptional control of genes by poly(A) tail removal of target mRNAs prior to their degradation. Here we show that *curled* encodes Drosophila Nocturnin and that the gene is required at pupal stage for proper wing morphogenesis after eclosion of the fly. Despite the complex ontogenetic expression pattern of the gene, *curled* is not expressed in the developing wing, and wing-specific *curled* knockdown mediated by RNAi does not result in the curled wing phenotype, indicating a tissue-nonautonomous, systemic mode of *curled* gene function. Our study not only presents an entry point into the functional analysis of invertebrate nocturnins but also paves the way for the identification of the still elusive Nocturnin target mRNAs by genetic suppressor screens on the *curled* wing phenotype.

ON December 15th, 1915 Thomas H. Morgan described the first *curled (cu)* mutant *Drosophila melanogaster* (Bridges and Morgan 1923). The posterior wing part of *curled* mutant flies is bent upwards, an eponymous phenotype, which made *curled* the founding member of a series of recessive or dominant marker mutations. They include *curled on X* (Krivshenko 1958), *curl* (Goldschmidt 1944), *curlex* (Lindsley and Grell 1968), *Curled 3* (Meyer 1952), *Curlyoid* (Curry 1939), *Curly* (Ward 1923), and *Upturned* (Ball 1935). *Curly* and *curled* are among the most popular wing marker mutations for research and are used daily in Drosophila research laboratories worldwide.

Despite the widespread use of curled wing mutants the morphogenetic cause of this phenotype is unclear. It has been proposed that curled wings result from contraction differences between the dorsal and ventral wing surfaces while the expanded wings dry after eclosion (Waddington 1940) and there are indications for a functional interrelationship among the curled winged phenotype mutants since *curled* is incompletely dominant in *Curly* mutants (Nozawa 1956a). Since similar wing phenotypes have also been described for *D. pseudoobscura* and *D. montium* mutants (Sturtevant and Novitski 1941), the mechanism underlying curled wing formation is likely to be evolutionarily conserved among Drosophilids. Moreover, the phenotypic expressivity of different curled wing mutants is variable depending on environmental factors such as larval nutrition during defined ontogenetic stages (Nozawa 1956a,b). Finally, the expressivity of the *curled* mutant wing phenotype has a cold-sensitive phase during late pupal stage (Nozawa 1956a). However, despite a long history of curled wing mutants none of the curled wing marker genes has been molecularly identified to date.

Recently we performed a genomewide screen to identify and characterize starvation-responsive genes in adult Drosophila flies (Grönke *et al.* 2005). Among the starvation-induced genes was the fly homolog of the vertebrate circadian rhythm effector gene *nocturnin* (*no; CG31299*), originally described as circadian rhythm gene in *Xenopus laevis* retinal photoreceptor cells (Green and Besharse 1996). Various other vertebrate nocturnins exert a circadian expression mode, most prominently in the mouse liver (Wang *et al.* 2001; Barbot *et al.* 2002) and in the human hepatoma cell line Huh7 (Li *et al.* 2008). In nocturnin knockout mice the

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central clock is unaffected but the mutant animals develop severe metabolic phenotypes due to impaired lipid uptake or utilization causing resistance to diet-induced obesity and hepatic steatosis (Green *et al.* 2007).

Nocturnin genes encode evolutionarily highly conserved members of a subfamily of the yCCR4-related protein family (Dupressoir *et al.* 2001), named after yeast Carbon Catabolite Repressor 4 (CCR4) (Denis 1984). All yCCR4-related proteins encode a C-terminal Mg^{2+}-dependent endonuclease-like domain (endo/exonuclease/phosphatase domain; pfam03372) (Dupressoir *et al.* 2001). The yCCR4-related protein family consists of four distinct subfamilies named after the proteins Nocturnin, Angel, 3635, and CCR4. The CCR4 subfamily proteins are catalytic components of a major cytoplasmic deadenylation complex in eukaryotes initiating mRNA decay by exonucleolytic 3′-5′ poly(A) tail removal (Chen *et al.* 2002). Like the yeast and vertebrate orthologs, the Drosophila CCR4 protein, which is encoded by the gene *twin*, acts as deadenylase on a number of different specific target mRNAs during embryogenesis and oogenesis as well as during heat-shock recovery (Temme *et al.* 2004; Morris *et al.* 2005; Semotok *et al.* 2005; Bönisch *et al.* 2007; Chicoine *et al.* 2007; Kadyrova *et al.* 2007). As yet the function of the Drosophila yCCR4-related protein subfamily representatives Angel and 3635 has not been studied.

Vertebrate Nocturnin proteins also exert deadenylase activity *in vitro* (Baggs and Green 2003; Garbarino-Pico *et al.* 2007) and are proposed to act in post-transcriptional regulation of hitherto unidentified target transcripts. As well as their pronounced circadian rhythm expression the vertebrate nocturnins are subject to clock-independent regulation implying additional and clock-independent functions in embryogenesis and/or metabolism. For example, Xenopus nocturnin is dynamically expressed from neurula stage onwards in various differentiating organs prior to the onset of the endogenous clock (Curran *et al.* 2008) and acute regulation of mouse nocturnin by physiological cues has been proposed. Moreover, nocturnin is an immediate early response gene of NIH3T3 fibroblast cells, characterized by its fast mRNA and protein turnovers (Garbarino-Pico *et al.* 2007).

Here we show, more than nine decades after the original description of the Drosophila gene *curled*, that the gene is identical to the fly ortholog of nocturnin. Uncovering the molecular identity of *curled* not only provides an entry point for the functional understanding of the prominent wing mutant phenotype, widely used as a marker in genetic studies, but also sets the stage for the analysis of the implications of fly *nocturnin* in circadian rhythm effector control and/or metabolism.

We show that Drosophila *curled(nocturnin)* has a highly complex and dynamic ontogenetic expression pattern and that the gene is acutely regulated upon physiological challenge. The Curled protein features all amino acids essential for catalytic function of CCR4 proteins and is localized in the cytoplasm, consistent with a possible function in mRNA decay via deadenylation as reported for yeast and vertebrates. We demonstrate that *curled (nocturnin)* is necessary during a narrow time window at late pupal stage for proper wing morphogenesis in adult flies and that this requirement is not tissue autonomous. Our results suggest that timely post-transcriptional regulation of effector genes is essential for proper wing formation during the wing expansion phase early after the eclosion of flies. Thus the *curled* mutant wing phenotype presents the first example of a morphogenetic function for a *nocturnin* gene family member that acts in the context of the long described Drosophila curled wing marker genes.

MATERIALS AND METHODS

Fly techniques: Flies were propagated at 25° on a complex corn flour-soy flour-molasses medium as described (Grönke *et al.* 2005). Fly strains used are showed in Table 1.

Heat-shock-induced *cu(no) in vivo* knockdown was induced by exposing the F_1 progeny of the cross *hs-GAL4* × *UAS-cu(no)* dsRNA (raised at 25°) for 20 min to 37° at the indicated time points/developmental stages. Identically treated F_1 progeny of w^{1118} × *UAS-cu(no)* dsRNA flies were used as controls.

Generation of *nocturnin* mutants: $w^*; P\{w^{+mC}=EP\}no(cu)^{GE22476}$ flies that carry an EP transposon-construct integration in the 5′ upstream region of the *nocturnin* gene at chromosome 3R between positions 7025884/5 (FlyBase *D. melanogaster* Genome Release 5.17), corresponding to position −2956 relative to the putative *no(cu)* start ATG in *no(cu)*-RD exon 1, were obtained from GenExel (Korea). *No(cu)* deletion mutants were generated by a conventional *P*-element mobilization scheme resulting in the small no^3 deletion (class I event) and the two larger deletions no^1 and no^2 (class II events) as well as the precise excision allele $no^{GE22476rv}$, which serves as a genetically matched background control. Sequencing of the relevant part of the *no(cu)* gene showed that no^3 and no^2 deletion mutants lack genomic DNA sequences from 3R: 7.025.888–7.027.112 and 7.025.888–7.032.973, respectively, corresponding to position (pos.) −2953 to −1729 and −2953 to +4132 relative to the putative *no(cu)* start ATG in *no(cu)*-RD exon 1. No^1 contains residual *P*-element sequence, which impeded the molecular characterization at the sequence level, but PCR analysis mapped the 3′ breakpoint between *no(cu)*-RC exon 1 and exon 6 (Figure 1A).

Fecundity assay and imaginal hatching rate: $cu^3(no^1)$ and $cu^4(no^2)$ mutants were crossed into a w^{1118} mutant background for five generations and reestablished as homozygous stocks. Embryos of the appropriate parental mutant and control genotypes (see Figure S1, A) were collected and mixed embryo collections were seeded in propagation vials to ensure identical propagation conditions. After hatching virgin females and males of the respective mutant or control genotypes were batch mated 1–2 days after hatching for 27 hr. Single females were then housed with 1–2 males and daily transferred to new food vials. Progeny were counted as embryos; the developmental speed was monitored and empty pupal cases counted to assess the percentage of imaginal hatching. For quantitative analysis females producing no progeny or not surviving the full observation period were excluded. Progeny values of all other females of a given genotype were averaged and the standard deviation calculated.

TABLE 1

Fly strains

Name (stock number)	Genotype	Reference/source
tubulin-GAL4 (RKF1057)	*w*; P{w^{+mC}=tubP-GAL4}LL7/TM3, P{w^{+mC}=ActGFP}JMR2, Ser^{1}*	BDSC no. 5138 (rebalanced)
Df(3R)M86D (RKF1063)	*Df(3R)M86D, Dfd[1] p[p]/TM3, Ser[1]*	BDSC no. 1714
FB+SNS GAL4 (RKF125)	*w*; P{w$^{+mW.hs}$=GawB}FB+SNS*	Grönke *et al.* (2003)
cu(no)^{GE22476} (SGF706)	*w*; P{w^{+mC}=EP}no^{GE22476} renamed to w*; P{w^{+mC}=EP}cu^{GE22476}*	GenExel
no^{1} or *cu^{3}* (SGF707)	*w*; no^{1} renamed to w*; cu^{3}*	This study
no^{2} or *cu^{4}* (SGF708)	*w*; no^{2} renamed to w*; cu^{4}*	This study
no^{3} or *cu^{5}* (SGF709)	*w*; no^{3} renamed to w*; cu^{5}*	This study
no^{GE22476rv} or *cu^{GE22476rv}* (SGF710)	*w*; no^{GE22476rv} renamed to w*; cu^{GE22476rv}*	This study
cu(no)$^{+12.2}$; cu^{3}(no^{1}) (SGF713)	*w*; P{w^{+mC}=CaSpeR4 cu(no)$^{+12.2}$}#43a/+; cu^{3}(no^{1})*	This study
no(cu)$^{stop12.2}$; cu^{3}(no^{1}) (RKF1067)	*w*; P{w+ mC=CaSpeR4 no$^{stop12.2}$}#20a; cu^{3}(no^{1})*	This study
UAS-cu(no)-RC:EGFP (SGF811)	*w*; P{w^{+mC} curled(nocturnin)[Scer\UAS]=UAS-cu(no)-RC:EGFP}#103/CyO float*	This study
UAS-cu(no)-RC:EGFP; *cu^{3}(no^{1})* (RKF1087)	*w*; P{w^{+mC} curled(nocturnin)[Scer\UAS]=UAS-cu(no)-RC:EGFP}#103; cu^{3}(no^{1})*	This study
UAS-cu(no)-RE:EGFP (SGF813)	*w*; P{w^{+mC} curled(nocturnin)[Scer\UAS]=UAS-cu(no)-RC:EGFP}#30/CyO float*	This study
UAS-cu(no)-RE:EGFP; *cu^{3}(no^{1})* (RKF1085)	*w*; P{w^{+mC} curled(nocturnin)[Scer\UAS]=UAS-cu(no)-RC:EGFP}#30; cu^{3}(no^{1})*	This study
cu^{1} (RKF1061)	*cu^{1}*	BDSC no. 468
cu^{1}/TM3, Sb^{1}	*ru^{1}, pbl^{5}, h^{1}, th^{1}, st^{1}, cu^{1}, sr^{1}, e^{s} ca^{1}/TM3, Sb^{1}*	BDSC no. 2452
TM6C, cu^{1}	*ash^{1}, B^{1}/TM6C, cu^{1}, Sb^{1}, ca^{1}*	BDSC no. 5045
cu^{2}/cu^{1} (RKF1062)	*In(3L)A54, st^{1} cu^{2} p^{p} red^{1} e^{s}/TM6C, cu^{1} Sb^{1} Tb^{1}*	BDSC no. 6591
Lsp2-GAL4 (RKF491)	*y^{1},w^{1118}; P{Lsp2-GAL4.H}3*	BDSC no. 6357
UAS-cu(no) dsRNA	*w^{1118}; P{GD8898}v45442*	Dietzl *et al.* (2007)
hs-GAL4 (GÖ432)	*P{GAL4$^{hs.2sev}$ry$^{p+t7.2}$=GAL4-Hsp70.sev}K25 (on 3rd)*	Ruberte *et al.* (1995)
w^{1118}	*w^{1118}*	This study

For additional strains see Table 3.

Molecular biology: Oligonucleotide primers used in this study are shown in Table 2.

cDNA isolation and transgene cloning: *cu(no)* and *SdhC* cDNAs were PCR amplified from an embryonic (0–22 hr) Drosophila Oregon R cDNA library. *cu(no)-RD* (pos. 75–2024 of NM_001104276.1) encoding full-length CU(NO)-PD was amplified using SGO363/SGO366 and cloned into pCRII-TOPO (www.invitrogen.com) resulting in pCRII-TOPO *cu(no)-RD* (SG261). *Cu(no)-RC* (pos. 1–1349 of NM_001104275; silent substitutions at pos. T411C and G1257A) encoding full-length CU(NO)-PC was amplified using SGO364/SGO367 and cloned into pEGFP-N2 (www.clontech.com). The *cu(no)-RC*:EGFP fusion cassette of the resulting pEGFP-N2 *cu(no)-RC*:EGFP clone (SG264) was subcloned *Bgl*II/*Not*I into pUAST (Brand and Perrimon 1993) resulting in pUAST *cu(no)-RC*:EGFP (SG268). Using the same strategy, *cu(no)-RE* (pos. 339–1680 of NM_001104277.1) encoding full-length CU(NO)-PE was amplified using SGO365/SGO367, cloned into pEGFP-N2 (resulting in pEGFP-N2 *cu(no)-RE*:EGFP clone (SG265) and the *cu(no)-RE*:EGFP fusion cassette subcloned into pUAST resulting in pUAST *cu(no)-RE*:EGFP (SG269). *SdhC* cDNA (pos. 2–596 of NM_141790.2) was amplified using SGO418/SGO419 and cloned into pCRII-TOPO (www.invitrogen.com) resulting in pCRII-TOPO *SdhC* (SG283).

The *no$^{+12.2}$* genomic rescue construct was generated in a three-step cloning process. First two *cu(no)* genomic DNA fragments were PCR amplified using the primer pairs SGO451/SGO454 and SGO452/SGO453 and cloned into vector pBluescript II KS(+) (www.stratagene.com) using the restriction sites indicated above resulting in pBS II KS(+) *cu(no)* 4.6 (SG296) and pBS II KS(+) *cu(no)* 7.6 (SG300), respectively. Subsequently, the SG300 DNA insert was released by *Xho*I/*Kpn*I restriction, cloned into equally restricted SG296, and the resulting 12.2-kbp genomic fragment subcloned via *Not*I/*Kpn*I restriction into pCaSpeR4 vector (https://dgrc.cgb.indiana.edu) to generate pCaSpeR4 *cu(no)$^{+12.2}$* (SG301).

A C->A nonsense mutation at *cu(no)-RE* position 1545 in the inactivated genomic rescue construct *cu(no)$^{stop12.2}$* was introduced by PCR using primer pairs SGO455/SGO457 and SGO452/SGO456 on the SG300 template. The two resulting DNA fragments were mixed, used as PCR template with primers SGO455/SGO452 and the PCR product cloned via *Xba*I/*Kpn*I to generate pBS II KS(+) *cu(no)^{stop}* 7.6. The subsequent generation of pCaSpeR4 *cu(no)$^{stop12.2}$* (SG302) followed the strategy outlined for SG301. Introduction of the mutation was confirmed by sequencing. Transgenic fly strains were established by *P*-element-mediated germ-line transformation as described (Grönke *et al.* 2003).

Identification of *curled* mutations: PCR-based *cu* gene locus analysis was performed with the following primer combinations (see Figure 2B): Amplicon a, SGO374/SGO375; amplicon b, SGO363/SGO391; and amplicon c, SGO405/SGO406. Control amplicon d was amplified from the *brummer* gene locus using SGO163/SGO209. Bloomington Drosophila Stock Center (BDSC) fly strains no. 2452, no. 5045 (for *cu^{1}* allele), and no. 6591 (for *cu^{2}* allele) were crossed to transcript null *no^{1}(cu^{3})* mutant flies. *Nocturnin(curled)* cDNAs of transheterozygous *cu^{1}/ no^{1}(cu^{3})* or *cu^{2}/ no^{1}(cu^{3})* flies were isolated as above

TABLE 2

Oligonucleotide primers

Name (stock number)	Sequence	Restriction site/ comment
SGO163	TGCCCTGTGAGAAGTGTAGA	
SGO209	GGGGCGTCTAATGTTATG	
SGO363	GGTAGATCTTAAAAGTGACAATGGATC	*Bgl*II
SGO364	CCTTAGATCTTGCCGCAGACATGGAG	*Bgl*II
SGO365	GCACAAAAAGATCTAAATGGAGTTTC	*Bgl*II
SGO366	TCGGCAAATTAGGTACCTGAAGCTTT	*Kpn*I
SGO367	TGGTCGACTGTATTGAATGGATCCATGCTTG	*Sal*I
SGO374	GTCATTGGCATTGTTATCAG	
SGO375	CCGAGGTGGCGTCTAATC	
SGO390	TCAGATCTCCAGAGCATTTGAACCGC	
SGO391	GCGGCTCGAACTCAAACTC	
SGO405	TGAACTGAATCCCCCATCTAA	
SGO406	GTTTGTTGTGTTACGCAATCC	
SGO418	CAGTCAGTGTACGGTCCCA	
SGO419	AATGTAATAGGTTTAGCTGGGG	
SGO451	GTGCGGCCGCAGCCTTACACGAATGTCG	*Not*I
SGO452	ATGGTACCTGTTGCTTTGGGCGTGCTGC	*Kpn*I
SGO453	GGTGACTCGAGGTTTATAGG	*Xho*I
SGO454	CCTATAAACCTCGAGTCACC	*Xho*I
SGO455	CAGATCAAAAACTGTCTAGACT	*Xba*I
SGO456	CCAATATCCAT**A**GGATCACTTTTC	Mutation in bold
SGO457	GAAAAGTGATCC**T**ATGGATATTGG	Mutation in bold
RKO681	GCGAAGAGGGCGAGGAATGTCA	
RKO682	TGGGTGTGCGATTCTTGCCAA	
RKO692	CGATATGCTAAGCTGTCGCACA	
RKO693	CGCTTGTTCGATCCGTAACC	

using the primer pair SGO390/SGO366, cloned into the pCRII-TOPO vector (www.invitrogen.com) and sequenced. *Cu(no)* mutations detected in the cu^1 allele of both cu^1 mutant fly lines or in differently sized cDNAs from $cu^2/no^1(cu^3)$ flies were confirmed by PCR amplification and sequencing of the corresponding genomic DNA sequence.

Northern blot and qRT–PCR: Total RNA for qRT–PCR analysis was prepared using peqGOLD TriFast reagent (www.peqlab.de) and reverse transcribed using the QIAGEN QuantiTect Reverse Transcription kit (www.qiagen.com). Real-time PCR analysis was performed on an Applied Biosystems StepOnePlus System using Applied Biosystems Fast SYBR Green Master Mix (www.appliedbiosystems.com) with the following primer pairs: *cu(no)*, RKO681/RKO682 and *RpL32*, RKO692/RKO693. Samples were analyzed in triplicate and experiments were repeated twice. Details are available on request.

Developmental Northern blot and quantitative Northern blot analysis were done as described (Grönke *et al.* 2003, 2005). In brief, Northern blots were prepared using the Northern Max kit (www.ambion.com) with 2 μg poly-A+ mRNA per lane for developmental and 10 μg total RNA per lane for quantitative expression analysis, respectively. Blots were successively hybridized with radioactively labeled *cu(no)* and *SdhC* antisense RNA probes using the Strip-EZ RNA kit (www.ambion.com). The universal *cu(no)* probe, detecting all annotated transcript isoforms, was generated by *in vitro* transcription of *Not*I linearized pCRII-TOPO *cu(no)-RD* (SG261), the *SdhC* probe of *Not*I linearized pCRII-TOPO *SdhC* (SG283) using Sp6 RNA polymerase. For quantification hybridized blots were scanned with a PhosphoImager (Fujifilm BAS; www.fujifilm.com) and signal intensity was quantified using AIDA Image Analyzer software v2.11 (www.raytest.de).

***In silico* methods:** Insect Nocturnin (Curled) homologs from *D. pseudoobscura* and *Anopheles gambiae* were identified in a tBlastN homology search (www.flybase.org) with *D. melanogaster* CU-PE and subsequently hand assembled. The deduced protein sequences were aligned with *D. melanogaster* CU-PE (ABW08641), the vertebrate Nocturnin homologs from *X. laevis* (AAB39495), *Mus musculus* (AAG01384), and *Homo sapiens* (Q9UK39) using the ClustalW algorithm of MEG-ALIGN (www.dnastar.com) to generate the Nocturnin protein alignment. The same alignment including in addition the *D. melanogaster* Twin (CG31137-PA, ACL89247), *D. melanogaster* Angel-PA (AAF47045), and *D. melanogaster* 3635 proteins (CG31759-PC, AAN10808) was used to generate the phylogenetic tree.

Imaging: *In situ* hybridization on embryos and third instar larval tissue using a digoxigenin-labeled RNA antisense probe was done as described (Grönke *et al.* 2003). The *cu* antisense probe was generated by *in vitro* transcription on *Not*I linearized pCRII-TOPO *cu-RD* (SG261) using Sp6 RNA polymerase (www.fermentas.com) and the DIG RNA labeling kit (www.roche-applied-science.com).

Wing and bristle phenotypes of anesthetized adult flies at days 4–6 posteclosion were imaged using a Zeiss Discovery V8 stereomicroscope equipped with a Qimaging Micropublisher 5.0 RTV camera. The same setup was used to record the early posteclosion wing expansion phase of $w^{1118};cu^3/cu^4$ (File S1) and w^{1118} (File S2) flies.

For *ex vivo* EGFP fluorescence detection fat body tissue of third instar larval progeny of the cross *Lsp-2-GAL4* × *UAS-no-*

RC:EGFP was hand dissected, embedded in phosphate-buffered saline, and imaged within 1 hr after preparation using a Leica TCS SP2 confocal microscope using 488 nm excitation and 500–541 nm emission wavelengths or transmission mode.

RESULTS

Molecular organization of the Drosophila *nocturnin* gene and generation of *nocturnin* mutants: In a screen for starvation-responsive genes in adult flies (Grönke *et al.* 2005), we identified Drosophila *nocturnin (no)* which was previously characterized as *bona fide* ortholog of the mammalian nocturnin genes by sequence alignment (Dupressoir *et al.* 2001). The *no* gene locus maps genetically at 86D7 on the third chromosome (genomic sequence annotation 3R 7.026.138–7.034.357) (Tweedie *et al.* 2009). It codes for three predicted *no* transcript isoforms (*no-RC*, *no-RD*, and *no-RE*) (Tweedie *et al.* 2009; see also Figure 1A) the existence of which was confirmed by cDNA isolation and sequencing. Conceptual translation of the *no* transcript isoforms predicts three different *no* proteins (NO), NO-PC, NO-PD, and NO-PE, which share a C-terminal Mg^{2+}-dependent endonuclease-like domain (pfam03372; NO-PE amino acids 115–411; Figure 1, A and B; see also Dupressoir *et al.* 2001) encoded by the common last five exons. Protein sequence alignment of *D. melanogaster* NO-PE to the other three fly yCCR4-related proteins called Twin, Angel, and 3635 as well as to Nocturnin proteins of other insects (*D. pseudoobscura*, *An. gambiae*) and of nonmammalian (*X. laevis*) as well as of mammalian vertebrates (*M. musculus*, *H. sapiens*) proves that NO is the *bona fide* Nocturnin ortholog of the fly (Figure 1, B and C and Dupressoir *et al.* 2001). Moreover it reveals remarkably high sequence conservation between Nocturnin proteins in particular in the putative Mg^{2+}-dependent endonuclease-like domain (between 53 and 89% sequence identity; Figure 1B). Notably all amino acids, which have been implicated in the domain's catalytic function, are completely sequence conserved. These findings suggest that Drosophila NO is a putative mRNA deadenylase involved in post-transcriptional regulation of target genes (see Figure 1B). Thus all three predicted Drosophila NO protein isoforms are likely to exert mRNA deadenylase function as reported for various yCCR4-related family proteins (Chen *et al.* 2002; Baggs and Green 2003; Morris *et al.* 2005).

To analyze *no* function *in vivo* we generated *no* deletion mutants by imprecise *P*-element excision of the *P{EP}GE22476* transgene construct, which is integrated immediately upstream of the *no* transcribed region (Figure 1A). Starting from the $no^{GE22476}$ fly strain the *no* alleles no^{1}, no^{2}, and no^{3} were isolated. They carry *no* deletions of different extent (see materials and methods). We also recovered the precise excision revertant $no^{GE22476rv}$, which served as a genetically matched control. *No* transcript expression in the deletion mutants was examined by Northern blot analysis (Figure 1D). Control flies express two prominent *no* transcript sizes of ~1.8 kb and 2.0 kb, referred to as S and L, respectively. The size of the annotated *no* transcripts (Figure 1A) suggests that the S band might correspond to *no-RC* (1529 bp) and *no-RE* (1856 bp), whereas the L band might correspond to *no-RD* (2194 bp). In no^{1} flies *no* S and L transcripts are absent, confirming that this mutation is indeed a transcript null allele. No^{2} mutants only express a low abundant *no* transcript, which is slightly smaller than the *no* S transcript of control animals. No^{3} mutant flies exclusively express *no* S transcripts. None of the *no* deletions affect the expression of the *no* downstream neighboring gene *SdhC* supporting the specificity of the *no* alleles (Figure 1D).

Homozygous flies carrying any of the three *no* deletions are viable and fertile. Whereas the wings of no^{3} mutant flies appear normal both the no^{1} and no^{2} mutants show a phenotype identical to *curled (cu)* mutants *i.e.*, upward bent (curled) wings and proximally crossed posterior scutellar bristles (Figure 1E). Since the *cu* gene has been genetically mapped to 86D3–86D4 (Tweedie *et al.* 2009), a chromosomal region very close to *no* at 86D7, we asked whether *no* and *cu* mutations could be alleles of the same gene.

***nocturnin* is *curled*:** To establish whether *cu* and *no* mutations affect the same gene, we performed a number of experiments that demonstrate that the curled wing phenotype of *no* mutants is caused by an inactivation of *no* and that the *nocturnin* gene is indeed encoded by the *curled* gene locus. Molecular data indicate that the no^{1} deletion is encompassed by deficiency *Df(3R)M86D* (Figure 2B), which genetically does not complement the curled wing phenotype of no^{1} nor of the cu^{1} or cu^{2} mutants (data not shown). Furthermore, no^{1}, cu^{1}, and cu^{2} failed to complement each other (Figure 2D; and data not shown). Moreover, the wing phenotype of homozygous no^{1} mutants can be completely reverted to wild type by the targeted expression of a NO-PC:EGFP or a NO-PE:EGFP fusion protein (Figure 2E and data not shown). Finally, the mutant phenotype of homozygous no^{1} flies as well as of transheterozygous no^{1}/cu^{1} mutant flies can be reverted by a 12.2-kbp genomic rescue transgene, termed $no^{+12.2}$, which spans the *no* gene locus (Figure 2, A and F, and data not shown).

The importance of the putative catalytic domain of NO is highlighted by the fact that a $no^{+12.2}$- derived control transgene termed $no^{stop12.2}$ fails to rescue the wing phenotype. This $no^{stop12.2}$ transgene carries a premature stop codon after amino acid position 401 of the NO-PE open reading frame resulting in a truncated NO protein that lacks part of the predicted endonuclease domain (Figures 1A and 2A) including evolutionarily conserved

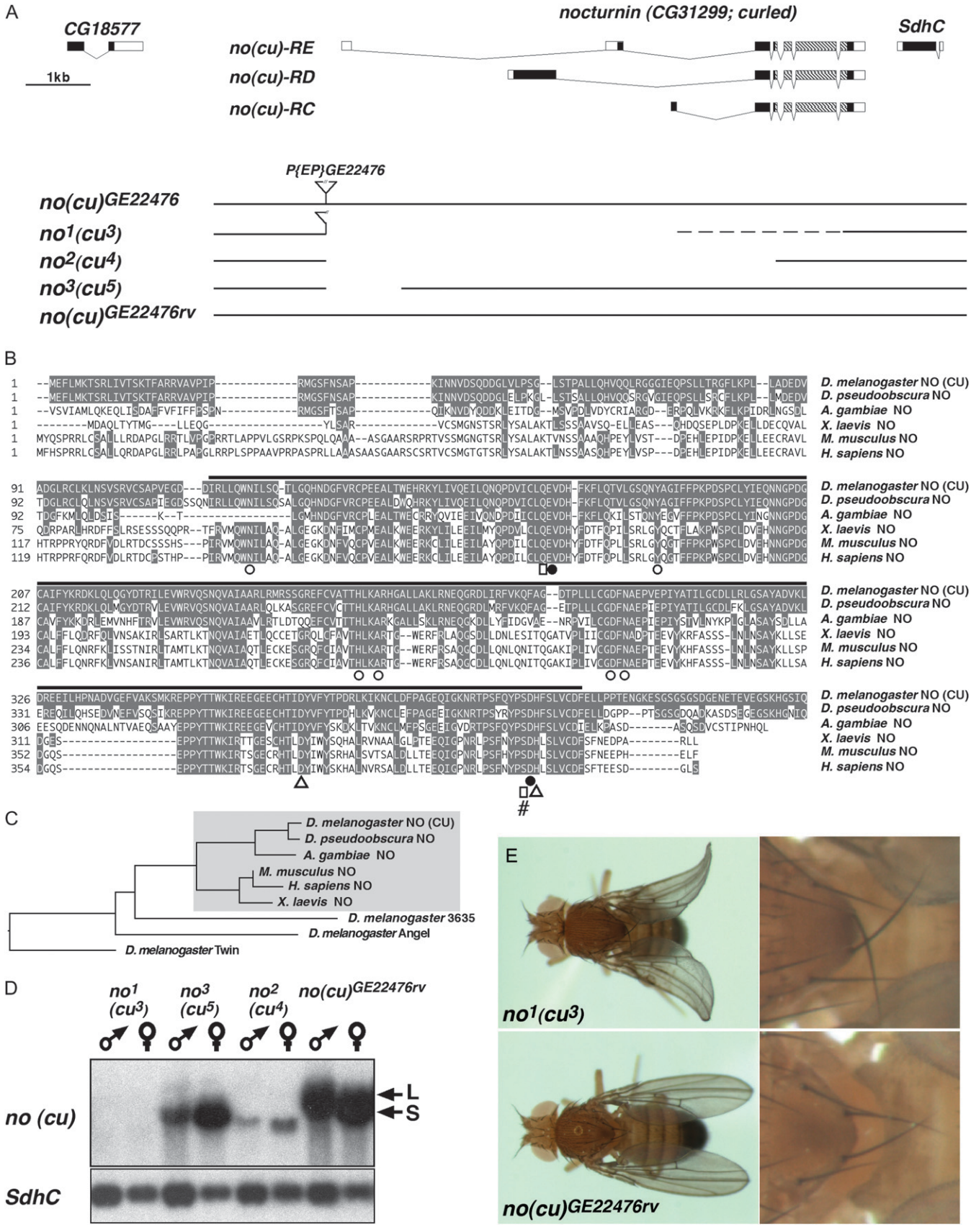

FIGURE 1.—The Drosophila *nocturnin (curled)* gene: gene locus organization, deletion mutants, and phylogeny of Nocturnin proteins. (A) Organization of *nocturnin (curled)* gene locus with transcript isoforms *no(cu)-RC*, *no(cu)-RD*, and *no(cu)-RE* relative to the flanking genes *CG18577* and *SdhC* at 86D7 on chromosome 3R. [Note: Coding parts of exons are marked by black, non-

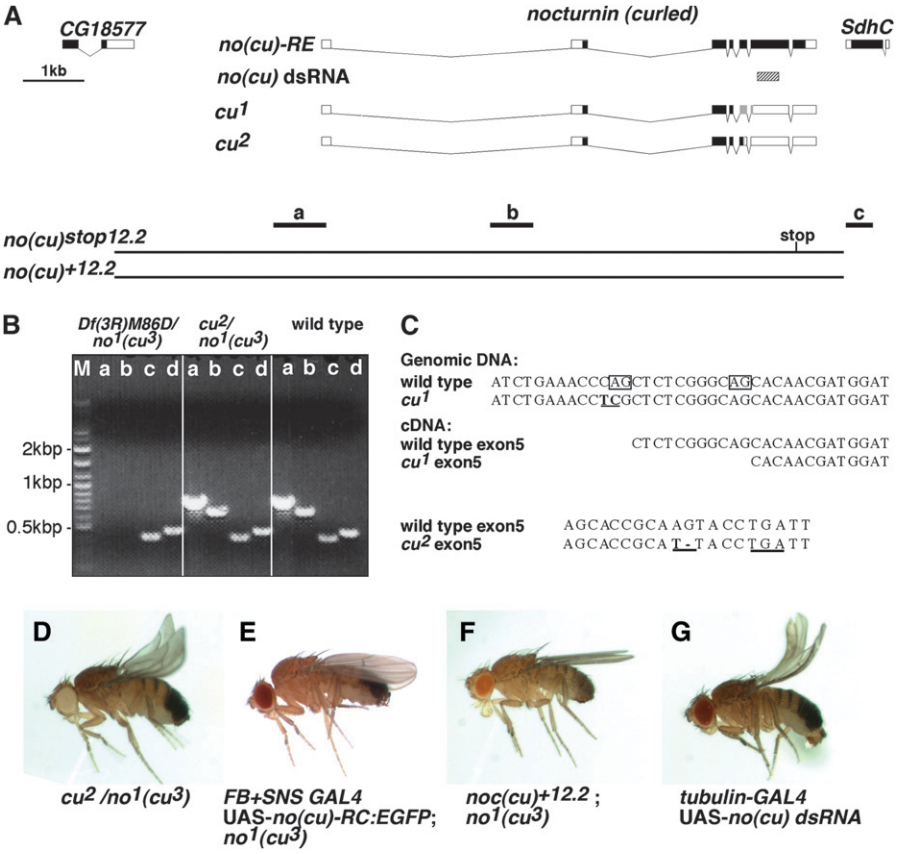

FIGURE 2.—curled is nocturnin. (A) Schematic overview of molecular lesions in the cu^1 and cu^2 alleles at the *nocturnin/curled* gene locus. Boxes represent noncoding (open), coding (black), or miscoding (shaded) exons with the exception of the hatched box [*cu(no)* dsRNA] representing the *cu(no)* coding region targeted by a transgenic dsRNA snapback construct. Extent of genomic *cu(no)* rescue constructs without [$cu(no)^{+12.2}$] and with [$cu(no)^{stop12.2}$] stop mutation in a functional essential region of Nocturnin. Bars labeled with a, b, and c indicate position and extent of PCR amplicons used in B. (Note: Bar d, control amplicon from the *brummer* locus; M, DNA molecular size marker.) PCR-based genotyping in B confirms coverage of no^1 (cu^3) by *Df(3R)M86D* and genomic integrity of *SdhC* in no^1 (cu^3) mutants. No gross *cu(no)* locus aberrations in the cu^2 mutant allele. (C) *cu(no)* splice site mutation in cu^1 and frameshift mutation in cu^2. The no^1 (cu^3) mutant wing phenotype is not complemented by cu^2 (D), and can be rescued by tissue-specific *cu(no)* cDNA (E), or $cu(no)^{+12.2}$ genomic (F) transgene expression. (G) *cu(no)* mutant phenocopy by ubiquitous expression of a *cu(no)* dsRNA transgene.

and functionally important amino acid residues essential for nocturnin protein function (Figure 1B). Taken together, these results unambiguously establish that a deletion of the *no* gene causes a curled wing phenotype and that the endonuclease domain is important for *no* gene function.

Molecular analysis of cu^2/no^1 transheterozygotes detected no gross molecular lesion of the X-ray induced cu^2 allele (Figure 2B). However, genomic and cDNA sequence analysis revealed that *no* transcripts are indeed affected in cu^1 and cu^2 mutants. Cu^1 mutants carry a splice acceptor site mutation at *no-RE* exon 5, which causes an alternative splice site usage. This leads to an 11-bp deletion followed by both a frameshift in the open reading frame and a premature translational stop signal (Figure 2, A and C). Similarly, cu^2 mutants carry a base pair substitution followed by a single-base-pair deletion at positions 67/68 of the *no-RE* exon 5, which cause a premature translational stop at amino acid position 147 of NO-PE (Figure 2, A and C). Accordingly, the NO open reading frame of the two independently generated *cu* mutant alleles lack the conserved endonuclease domain. Thus, cu^1 and cu^2 appear to be loss-of-function alleles.

Thus, we provide ample evidence that *nocturnin* is identical to the previously described gene *curled* for which reason the latter name will be maintained. Therefore, we refer in the following text to no^1, no^2,

coding parts by white boxes; hatched boxes, Mg^{2+}-dependent endonuclease-like domain (pfam03372); *no(cu)-RC, -RD, -RE* are the only *no(cu)* transcripts annotated in FlyBase r5.17.] Transposon integration line $P\{EP\}no(cu)^{GE22476}$ was used to generate *nocturnin (curled)* deletion mutants no^1 (cu^3), no^2 (cu^4), and no^3 (cu^5) as well as genetically matched no^+ (cu^+) control line $no(cu)^{GE22476rv}$. (B) Sequence alignment of vertebrate und invertebrate Nocturnin (NO) protein family members proves strong evolutionary conservation of amino acids involved in the catalytic function of yCCR4-related family proteins (amino acids identical to *D. melanogaster* NO are shaded in gray). Residues essential for the Mg^{2+}-dependent endonuclease-like domain function are indicated as in DUPRESSOIR *et al.* (2001): △, catalytic residue; □, residues involved in orientation and stabilization of catalytic residues; ○, for phosphate binding, and ●, for Mg^{2+} binding residues; #, position of stop in $no^{stop12.2}$. The black bar illustrates the extent of the pfam03372 domain. (C) Phylogenetic tree analysis showing that Drosophila NO(CU) is the Nocturnin ortholog (Nocturnin subfamily shaded gray) among the four yCCR4-related proteins of the fly. (D) Northern blot analysis of adult flies shows *nocturnin (curled)* small S and large L transcript populations in $no(cu)^{GE22476rv}$ controls and identifies no^1 (cu^3) as transcript null mutation. No^3 (cu^5) specifically lacks *no(cu)* L transcripts, while no^2 (cu^4) expresses a low abundance transcript slightly shorter than the *no(cu)* S transcript of control flies. *SdhC* transcript is unaffected in all *no(cu)* mutants. (E) Distally upward bent wing (left panel) and proximally crossed posterior scutellar bristle (right panel) phenotypes of no^1 (cu^3) mutants compared to $no(cu)^{GE22476rv}$ controls.

and no^3 mutant alleles as cu^3, cu^4, and cu^5, respectively, to the transgene bearing flies described above as $cu^{+12.2}$ and $cu^{stop12.2}$ and finally to the EGFP containing fusion transgenes as UAS-*cu-RC:EGFP* and UAS-*cu-RE:EGFP*, respectively.

***curled* developmental expression and its relation to wing morphogenesis:** As observed with its vertebrate orthologs (Dupressoir *et al.* 1999; Wang *et al.* 2001; Curran *et al.* 2008) the Drosophila gene *cu* displays a complex expression pattern. Developmental Northern blot analysis revealed *cu* expression during all ontogenetic stages from early embryos to adult flies. The small *cu* transcript S observed during early embryogenesis is maternally provided. In all other stages of the Drosophila life cycle both the S and the L transcripts are expressed (Figure 3A). *In situ* hybridization detects strong *cu* transcript enrichment in embryonic salivary glands, the distal part of the proventriculus (posterior to the later imaginal ring region) and the ring gland as well as weak expression in the midgut (Figure 3B). The proventricular and ring gland *cu* expression domains are also found at third instar larval stage (Figure 3C). *Cu* transcript could not be detected by *in situ* hybridization in the larval central brain, in the imaginal discs, the salivary glands, or in the fat body (Figure 3C and data not shown). The latter result is in line with the absence of *cu* cDNAs in a larval fat body EST library (Jiang *et al.* 2005). However, microarray experiments detect weak *cu* expression in this organ (Chintapalli *et al.* 2007), which suggests low abundance *cu* expression in the fat body at the detection limit. Furthermore, *cu* expression shows neither gender specificity nor differences between head and body in adult flies (Figure 3A). Tissue-targeted *in vivo* expression of a functional CU-PC:EGFP fusion protein in larval fat body cells indicates that the protein is homogeneously distributed in the cytoplasm (Figure 3D) as has been reported for endogenous Nocturnin protein in Xenopus retinal photoreceptor cells (Baggs and Green 2003).

Collectively, the *cu* gene is expressed during all stages of the Drosophila life cycle, and transcripts are enriched in metabolically active tissues such as the ring gland, salivary gland, and the proventriculus. This expression profile of *cu* argues for specific developmental and/or metabolic functions of the CU protein. However, neither the reduction of *cu* gene function by a ubiquitous RNAi-mediated gene knockdown (see below) nor lack of the *cu* gene impairs the fecundity of adult flies (Figure S1, A). Additionally, *cu* mutants show a normal survival rate and developmental time during ontogenesis from embryos to adult flies (Figure S1, B; and data not shown). Thus, reduction or lack of *cu* function reveals no developmental role of the endogenous *cu* expression other than in adult wing morphogenesis. Notably, however, ectopic expression of the CU-PC:EGFP fusion protein under ubiquitous driver control causes lethality at pupal stage (data not shown). This result suggests that control of *cu* expression dosage is critical for normal fly development.

Upwardly bent, curled wings are the most prominent phenotype of mutant flies carrying a *cu* gene deletion or lacking an intact CU C-terminal endonuclease domain. Accordingly, it was surprising to find that the *cu* gene is not expressed during any stage of wing development. The discrepancy between the *cu* gene expression domains and the specific *cu* mutant wing phenotype argues therefore against a tissue-autonomous mode of *cu* gene action. To test this hypothesis we took advantage of tissue-specific *in vivo* gene knockdown in response to a UAS-*cu(no)* dsRNA transgene that was controlled by a variety of different GAL4 drivers using the GAL4/UAS system (Brand and Perrimon 1993). Ubiquitous *cu* knockdown forced by different driver constructs phenocopies the *cu* mutant wing phenotype (Figure 2G; and Table 3), whereas expression of the UAS-*cu(no)* dsRNA effector transgene in wing imaginal discs had no impact on wing morphogenesis (Table 3, Figure S1, E). Interestingly, *cu* knockdown in various different individual organs such as the nervous system, ring gland, muscles, fat body, tracheal system, salivary gland, and gut including each of the various endogenous *cu* expression domains was also insufficient to interfere with normal wing development (Table 3). These data suggest that the *cu* gene acts in a nonautonomous manner and that the endogenous expression domains of the gene have possibly redundant functions in providing *cu* activity. This conclusion gains further support by the finding that CU-PC:EGFP expression targeted to the fat body and the stomatogastric nervous system of *cu* mutants can fully rescue the wing phenotype (Figure 2E; note the absence of wing expression shown in Figure S1, D), whereas the *cu* knockdown by UAS-*cu(no)* dsRNA effector transgene expression in the same spatiotemporal expression patterns does not interfere with normal wing development (Table 3).

To determine the phenocritical period of *cu* requirement for normal wing development we performed *cu* knockdown experiments by ubiquitous UAS-*cu(no)* dsRNA expression pulses in response to a heat-shock-controlled GAL4 driver transgene. Ubiquitous *cu* knockdown at any ontogenetic stage prior to the third larval instar (L3) stage did not result in a curled wing phenotype (data not shown), whereas *cu* dsRNA expression pulses during mid and late L3 (96–120 hr preeclosion) caused curled wings in low penetrance (<30%; Figure 3E). A fully penetrant curled wing phenotype was obtained, however, when *cu* dsRNA pulses were expressed during the pupal stage. Remarkably, *cu* dsRNA expression as late as 11–24 hr prior to fly eclosion could still induce the curled wing phenotype in the majority of the individuals. Quantitative RT–PCR experiments indicate that the corresponding rapid phenotypic response correlates with a reduction of *cu* mRNA abundance by ~50% (Figure 3E). These findings show that the pupal stage is the

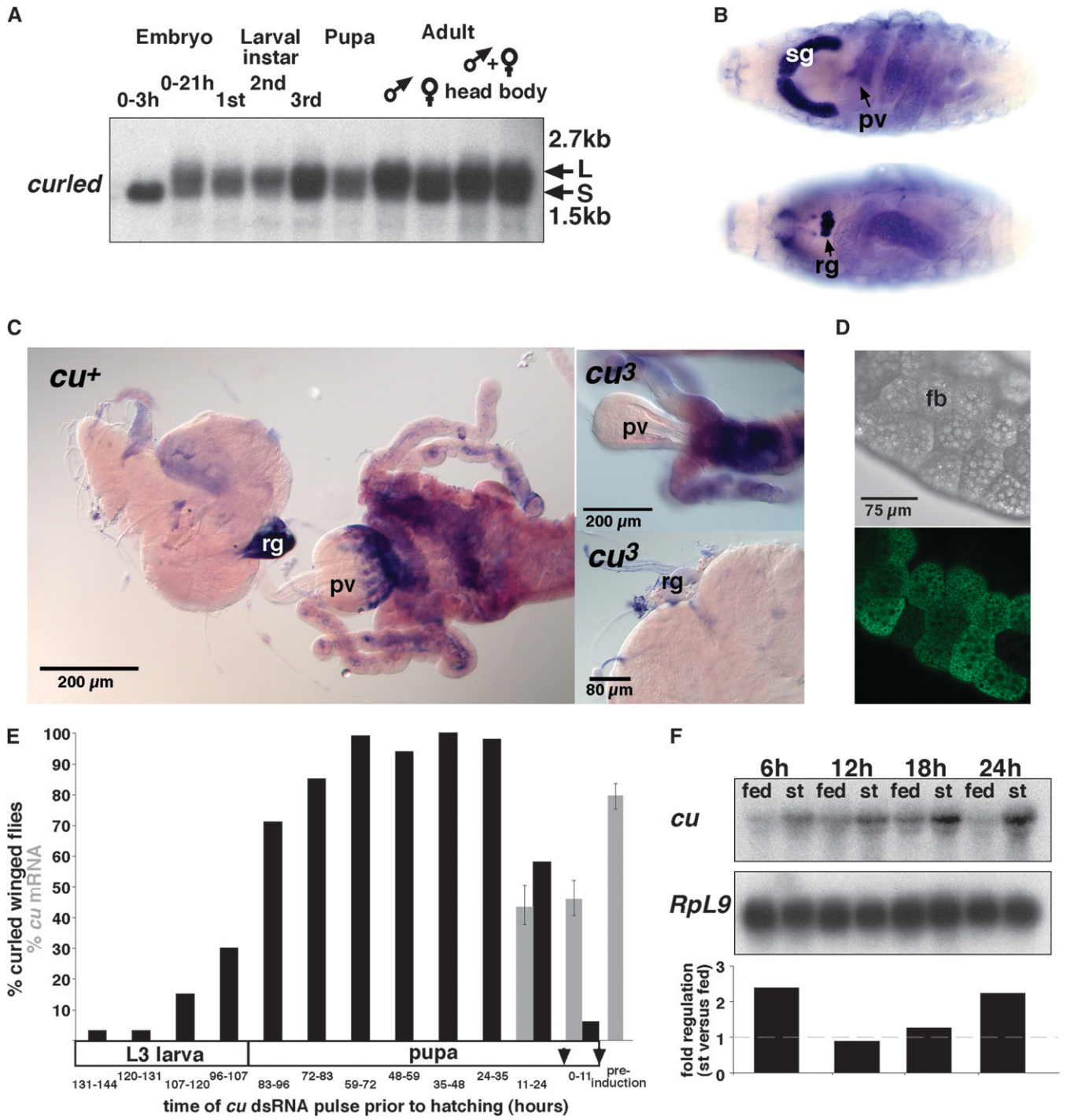

FIGURE 3.—Complex and dynamic *curled* developmental gene expression and pupal function for wing morphogenesis. (A) Developmental Northern blot analysis detects *curled* S (±1.8 kb) and L (± 2.0 kb) transcript populations at all ontogenetic stages with the exception of early embryos, exclusively expressing maternally contributed *cu* S transcripts. (B and C) Tissue-specific expression of *cu* transcripts in embryos and third instar larvae. Expression in embryonic salivary glands (B) and in embryonic (B) and third instar larval (C) proventriculus and ring gland absent from cu^3 deletion mutants. (D) Cytoplasmic intracellular CU-PC:EGFP localization upon targeted expression in third instar larval fat body. (Note exclusion from lipid droplets.) (E) Phenocritical period of *curled* wing morphogenesis function in pupae determined by *in vivo* RNAi. Percentage of curled winged phenotypes (black columns) and *cu* transcript expression levels (gray columns) scored in flies subjected to developmental time-controlled ubiquitous *cu* gene knockdown pulses during third instar larval and pupal development. Note: Arrows exemplify time points of heat-shock-mediated *cu* knockdown induction and error bars 95% confidence intervals. (F) Quantitative Northern blot analysis demonstrates starvation-responsive transcriptional upregulation of *cu* in adult male flies. sg, salivary gland; pv, proventriculus; rg, ring gland; st, starved.

TABLE 3

Tissue-specificity analysis suggests a systemic *cu* function for wing morphogenesis

Driver line genotype:	Driver line tissue specificity:	No. of F_1 scored:
Conditional *curled* knockdown causing curled wing phenotype		
w; P{w+mC=tubP-GAL4}LL7/TM3, P{w+mC=ActGFP}JMR2, Ser1* (1m)	Ubiquitous	>100
y1 w; P{w+mC=Act5C-GAL4}25FO1 / CyO, P{w+mC=ActGFP}JMR1* (1m)	Ubiquitous	>100
w; da-GAL4*	Ubiquitous	112[a]
Conditional *curled* knockdown causing no curled wing phenotype		
Driver line genotype:	Driver line tissue specificity:	No. of F_1 scored:
w; P{w+mW.hs=GawB}10* [c]	Wing disc	133
w; P{w+mW.hs=GawB}C-765* [d]	Wing disc	135
P{w+m=GAL4}A9, w**.[b]	Wing disc	>100
w; P{GAL4-vg.M}2; TM2/TM6B, Tb1* [b]	Wing disc	81
w1118 P{GawB-DeltaKE}Bx^MS1096-KE [b]	Wing disc	60
w; P{w+mW.hs=GawB}30A/CyO* [m]	Wing disc, eye-antennal disc, salivary gland	37
y [a] w [a]; P{w+mW.hs=GawB}FB P{w+m UAS-GFP 1010T2}; +/+* [e]	Fat body, salivary gland	>100
w; 3.1Lsp2-Gal4 line 2/TM3, Sb **.[f]	Fat body	54
y w*; r4-gal4* [g]	Fat body	61
w; yolk-Gal4 (II)* [j]	Female Fat body	64 (females)
w; P{w+mW.hs=GawB}FB+SNS* [e]	Fat body, stomatogastric nervous system, salivary gland	>100
w1118; P{w+mC=Sgs3-GAL4.PD}TP1 [b]	Salivary gland	>100
P{w+mW.hs=GawB}elav^C155 [b]	Nervous system	54
P{w+mW.hs=GAL4-Nrv2-3} P{w+m UAS-GFP}* [h]	Nervous system	55
w1118; P{w+mW.hs=GawB}drl^PGAL8 [b]	Primarily mushroom body and central body complexes	27
w; Burs-Gal4* [i]	Bursicon-positive neurons	44
y1 w; P{w+mC=GAL4-per.BS}3* [b]	*period*-positive neurons	65
w; P{w+mW.hs=GawB}dimm^929* [b]	Peptidergic neurons	69
w; P{w+mW.hs=GawB}386Y* [b]	Peptidergic neurons	55
y1 w; P{w+mC=Ccap-GAL4.P}16* [b]	CCAP-secreting cells of ventral ganglion and brain	87
P{w+mC=GAL4-Eh.2.4}C21 [b]	Eclosion hormone-expressing neurons	63
w1118; P{w+mC=drm-GAL4.7.1}1.1/TM3, Sb1 [b]	Embryonic proventriculus, anterior midgut, posterior midgut, Malpighian tubules, and small intestine	45
w1118; P{w+mW.hs=GawB}l(2)C805^C805/CyO [b]	Larva: ring gland, histoblasts, gut and Malpighian tubules Adult: male accessory glands, testis sheath and cyst cells	44
w; P{GAL4-btl.S}* [k]	Tracheal system	>100
P{GAL4-Mhc.W}MHC-82 [l]	Muscles	>100

Ubiquitous but no tissue-specific *cu* knockdown causes the curled wing phenotype. All driver lines were crossed against the *UAS-cu(no)* dsRNA effector line.

[a] expressivity low
[b] BDSC, (1m) Modified BDSC stock
[c] K. Basler laboratory
[d] Guillén *et al.* (1995)
[e] Grönke *et al.* (2003)
[f] Lazareva *et al.* (2007)
[g] Lee and Park (2004)
[h] Sun *et al.* (1999)
[i] Peabody *et al.* (2008)
[j] Georgel *et al.* (2001)
[k] Shiga *et al.* (1996)
[l] Schuster *et al.* (1996)
[m] Brand and Perrimon (1993)

phenocritical period of the *cu*-dependent wing phenotype and that a rapid decrease of *cu* mRNA abundance at eclosion can cause the phenotype observed.

The *cu* mutant wing phenotype can be suppressed by larval crowding and/or in malfed larvae (Nozawa 1956a,b) likely caused by riboflavin shortage during larval stages (Pavelka and Jindrák 2001). We therefore asked whether *cu* mRNA abundance could also be acutely modulated by altered physiological cues such as food deprivation. To demonstrate such a principal effect on *cu* mRNA, the *cu* transcript abundance in starved adult flies was monitored by quantitative Northern blot analysis (Figure 3F). The results show that *cu* transcripts quickly accumulate upon food deprivation (2.5-fold after 6 hr) and that the gene is dynamically regulated during an extended starvation period (Figure 3F; see also microarray data in Grönke *et al.* 2005). (Note: This work refers to the previous *nocturnin* gene annotation *CG4796*; FlyBase ID FBgn0037872.)

Thus, in agreement with previous studies concerning the modulation of the *cu* wing phenotype in response to environmental factors, *cu* transcript abundance is strongly affected by food deprivation.

Is *curled* involved in bursicon-controlled posteclosion behavior? Both aspects of the *cu* mutant phenotype, *i.e.*, the posterior upwardly bent wings as well as the misorientation of the posterior scutellar bristle, become manifest at expansion phase early after eclosion of the adult fly (File S1). During that stage, flies' behavior follows a stereotypical though environmentally modulated complex motor pattern (Fraenkel *et al.* 1984). The immediate posteclosion behavior is composed of two active motor phases (Baker and Truman 2002) called perch selection phase [phase I according to Peabody *et al.* (2009)] and expansion phase (phase III), which are separated by a largely sedentary interphase (phase II). The expansion phase is initiated by abdominal elongation and flexion of the body and eventually leads to wing and cuticle expansion (File S2). This stereotyped posteclosion behavior is coordinated by the neuropeptide bursicon (reviewed in Honegger *et al.* 2008), which is released into the hemolymph by a subset of N_{CCAP} neurons during posteclosion phase II (Luan *et al.* 2006; Peabody *et al.* 2008, 2009). Bursicon controls the phase III behavior but it also has somatic functions including cuticle tanning (Baker and Truman 2002; Dai *et al.* 2008) and in transient cuticle plasticization allowing for body and wing expansions (Reynolds 1976, 1977).

cu mutants show a normal phase I–III posteclosion behavior sequence suggesting that *cu* gene function is unlikely to be involved in the behavioral output of *bursicon* (*burs*) activity at the level of N_{CCAP} neurons. This conclusion is supported by the finding that *cu* knockdown in bursicon-positive N_{CCAP} neurons does not interfere with normal wing morphogenesis (Table 3). Moreover *cu* mutants tan properly (Figure S1, C and D). This observation argues against a putative *cu* function in *burs*-mediated body pigmentation. However, the fact that both aspects of the *cu* mutant phenotype become manifest during the bursicon-controlled phase III of posteclosion behavior still leaves the possibility that the requirements for both, the *burs* and the *cu* genes or their effectors are interconnected. Unfortunately, a direct comparison of the *cu* and *burs* mutant wing phenotype development is impossible since *burs* mutants fail to expand their wings (Dewey *et al.* 2004) and the details of the incomplete wing expansion sequence in flies subjected to activity modulation of bursicon-releasing N_{CCAP} neurons have not been reported (Luan *et al.* 2006). Thus, it is undecided whether the partially expanded wing development of those mutant flies follows the wild-type sequence, transiently adapting a downwardly cupped wing shape (Peabody *et al.* 2009) or whether the expanding wings bend up immediately as observed in *cu* mutants (compare File S1 and File S2). However, it is noteworthy that the posterior scutellar bristle reorientation, which is likely a consequence of thoracic cuticle expansion, fails not only in *cu* mutants but also in flies carrying the $burs^{Z1091}$ or the $burs^{Z1091}/burs^{Z5569}$ mutant alleles (Dewey *et al.* 2004). Accordingly, a model proposing that *cu* affects a somatic output of *burs* signaling which in turn causes structural or physiological changes in wing and thoracic cuticle plasticity cannot be excluded. In accordance with this model we found that *cu* acts neither cell autonomously in the wing tissue nor in an organ-specific manner since wing- or organ-specific *cu* knockdown failed to cause a curled wing phenotype (see above and Table 3).

DISCUSSION

Here we provide evidence that the Drosophila gene *cu*, initially discovered >90 years ago by Thomas H. Morgan (Bridges and Morgan 1923), encodes the fly ortholog of vertebrate Nocturnin. The proposal that *cu* encodes a deadenylase as described for Nocturnin is based on strong circumstantial evidence. In vertebrates as well as in Drosophila all four yCCR4-related protein subfamilies, which are named for CCR4, nocturnin, 3635, and Angel are each represented by a single ortholog of high sequence conservation (Dupressoir *et al.* 2001). CCR4 subfamily members from yeast to mammals including the fly CCR4 ortholog Twin have been demonstrated or proposed to act as the catalytic component of the cytoplasmic deadenylase, which exerts 3′–5′ poly(A) RNA exonuclease activity (Chen *et al.* 2002; Temme *et al.* 2004). Similarly, *in vitro* poly(A)-tail-specific exonuclease activity involved in deadenylation has been shown for both Xenopus and mouse Nocturnin (Baggs and Green 2003; Garbarino-Pico *et al.* 2007). Moreover, all catalytic residues critical for CCR4 enzymatic function are absolutely sequence conserved in Curled (Figure 1B; see also Dupressoir *et al.* 2001). Consistent with its

putative function in post-transcriptional mRNA control the EGFP-tagged CU is cytoplasmatically localized (Figure 3D) as has been observed for endogenous Xenopus Nocturnin (Baggs and Green 2003), a yeast Ccr4p–GFP fusion protein (Sheth and Parker 2003), the endogenous Drosophila CCR4 (Temme *et al.* 2004), an HA-tagged Drosophila CCR4 in nurse cells and blastoderm embryos (Lin *et al.* 2008), and a GFP-tagged hCcr4 in HEK293 cells (Cougot *et al.* 2004). These aspects of Drosophila Curled are in agreement with its proposed function in post-transcriptional control of target mRNAs via deadenylation.

Poly(A) tail removal is the first step in post-transcriptional gene repression followed by the decay of the corresponding mRNAs (reviewed in Meyer *et al.* 2004; Garbarino-Pico and Green 2007; Houseley and Tollervey 2009). Given the potent regulatory impact of deadenylases high selectivity of targeted mRNA species is required, possibly mediated by RNA-interacting proteins. To date no direct regulatory target mRNAs of any Nocturnin family protein is known but a yeast two-hybrid screen identified Quaking-related 58E-1 (Qkr58E-1) as an interaction partner of Nocturnin/Curled (Giot *et al.* 2003). This putative binding partner carries an RNA-binding K homology domain (Fyrberg *et al.* 1998) and thus may serve as mediator between Curled and specific target mRNAs.

The question of whether fly *cu* acts as a vertebrate nocturnin-like circadian cycling gene needs to be carefully addressed in future studies. Various genome-wide microarray-based studies have not identified *cu* as a cycling gene (Claridge-Chang *et al.* 2001; McDonald and Rosbash 2001; Ceriani *et al.* 2002; Lin *et al.* 2002; Ueda *et al.* 2002) nor has a subsequent metaanalysis that was based on the data sets of the aforementioned studies (Keegan *et al.* 2007). However, these studies assessed global cycling patterns based on RNA extracted from total fly heads or bodies and likely would have missed circadian cycling of genes expressed in specific tissues/organs under peripheral clock control. In fact, adult *cu* expression has been reported not only in the brain but also in carcass preparations housing the adult abdominal fat body and oenocytes, which execute adipose-tissue and liver-like functions, respectively (Butterworth *et al.* 1965; Gutierrez *et al.* 2007), as well as in malpighian tubules, the fly kidneys (Chintapalli *et al.* 2007). Peripheral clocks have been reported to operate in all three of these metabolic nodal points (Giebultowicz and Hege 1997; Hege *et al.* 1997; Krupp *et al.* 2008; Xu *et al.* 2008). Accordingly, an evolutionarily conserved metabolic effector gene function of Drosophila *cu* is a possibility to be explored.

Although the circadian expression and function of vertebrate nocturnins is well studied, experimental data also suggest an additional, noncircadian role for these nocturnins. In mouse and Xenopus the nocturnin genes are already expressed during early embryogenesis (Wang *et al.* 2001; Curran *et al.* 2008). Interestingly, in frog nocturnin expression domains precede the onset of circadian rhythm expression of the clock gene Bmal1 (Curran *et al.* 2008), implying clock-independent nocturnin functions at this stage of development. Similarly, mouse nocturnin expression during embryogenesis has been reported (Wang *et al.* 2001) and Drosophila *cu* displays a complex tissue-specific embryonic expression pattern but their significance remains elusive since both nocturnin knockout mice and the *cu* mutant flies are viable and fertile and undergo apparently normal ontogenesis (Green *et al.* 2007) (Figure S1, A and E).

A selective, modulatory role of nocturnin would be consistent with the complex, though relatively subtle, metabolic phenotypes of knockout mice where phenotype and lipometabolism gene expression are nutritionally regulated (Green *et al.* 2007). The question remains open whether the primary cause of the mouse knockout phenotypes is the lack of circadian nocturnin expression in liver and/or other tissues. Alternatively or additionally the failure of a potential second regulatory aspect of nocturnin could contribute to the phenotypes. In fact, nocturnin is an immediate early gene when murine NIH3T3 fibroblast cells are exposed to phorbol ester or serum stimulation and both nocturnin mRNA and protein are characterized by a short turnover rate (Garbarino-Pico *et al.* 2007). Thus the gene is acutely regulated by physiological cues. Our findings that *cu* mRNA accumulates rapidly upon starvation of flies (Figure 3E) and that flies show a very sensitive phenotypic response to an acute *cu* downregulation (Figure 3D) would be in line with these results in mammalian cells. Furthermore, *cu* is downregulated in Drosophila Schneider (S2) cells depleted for the negative elongation factor (NELF), a transcription regulatory complex that affects rapidly inducible genes by stalling of RNA polymerase II (Gilchrist *et al.* 2008). Notably, the *cu* gene in S2 cells shows a promotor-proximal enrichment of NELF subunits, of the GAGA factor (Lee *et al.* 2008) and of RNA polymerase II (Muse *et al.* 2007) proposed to be characteristic for rapidly inducible stimulus-responsive genes (Gilchrist *et al.* 2008). Consistently, RNA polymerase II stalling at the *cu* promotor has been demonstrated in fly embryos (Zeitlinger *et al.* 2007). Taken together, the current data on *cu* regulation portray a gene subject to acute and dynamic regulation and thus it shows characteristics reminiscent of the non-circadian regulatory aspects of its mouse ortholog.

Besides showing that the curled wing phenotype of *cu* mutants is due to the loss of Drosophila *nocturnin* expression, our results highlight the possibility that mutants affecting a putative deadenylase involved in mRNA degradation can act in a non-cellautonomous manner during posteclosion morphogenesis. This mode of action is well established for hormones, like bursicon, which is produced in few endocrine cells of the fly, and systemically affects posteclosion morphogenesis as soon

as it becomes available to all cells after its release into the hemolymph. But how can a putative enzyme that is located in the cytoplasm of cells outside the developing wing control proper wing morphogenesis? Taking all results presented in this study into account, we speculate that Drosophila Curled is required to degrade, among others, a specific mRNA which encodes a factor that prevents the synthesis or the conversion of a metabolic compound and/or its release into the hemolymph. In *cu* mutants this metabolic compound is either not synthesized or remains trapped in the cells that lack *cu* gene function and thus, it would not be available in target cells at the time when adult wing morphogenesis occurs. It appears that such a hypothetical compound can be supplied by many or all cells since the mutant wing phenotype can be only elicited by ubiquitous but not by tissue-specific *cu* gene knockdown. The disclosure of the molecular nature of such a compound and the question of whether and how such a component could interact with the activity of the bursicon pathway or whether it acts in parallel has to await the identification of first direct targets of Nocturnin proteins in vertebrates or Drosophila. The Drosophila *cu* mutant presents an ideal entry point for a genetic suppressor screen to identify such target genes.

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GENETICS

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curled Encodes the Drosophila Homolog of the Vertebrate Circadian Deadenylase Nocturnin

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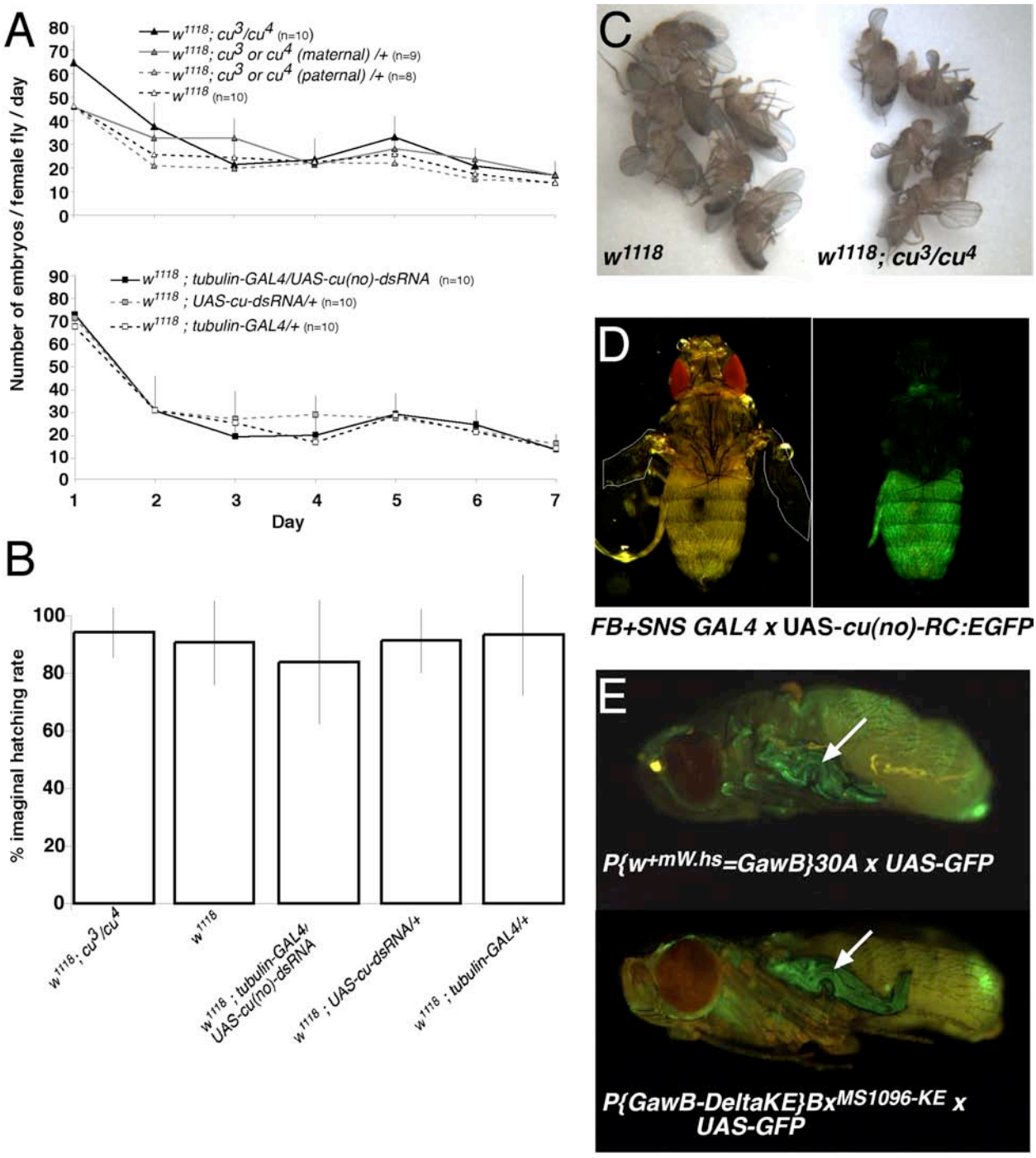

FIGURE S1.—Fecundity, imaginal hatching rate and pigmentation of *cu* mutant flies and absence of pupal wing expression. (A) Normal fecundity of cu^3/cu^4 mutant flies or females exhibiting a ubiquitous *cu* gene knockdown compared to the corresponding controls. Depicted are average number of embryos laid per female fly per day and the corresponding standard deviations. n refers to the number of females scored. (B) Comparable imaginal hatching rates between cu^3/cu^4 mutant flies or females exhibiting a ubiquitous *cu* gene knockdown compared to the corresponding controls. (C) Comparable body pigmentation of male w^{1118}; cu^3/cu^4 mutant and w^{1118} control flies 3.5 hours after hatching. (D) Absence of late pupal wing expression of CU-RC:EGFP targeted by the *FB+SNS GAL4* driver transgene, a combination, which rescues the *cu* mutant wing phenotype. Note: white lines outline pupal wings. (E) Late pupal wing expression (arrows) of a GFP reporter driven by two of the GAL4 lines which fail to cause curled mutant wings when combined with an UAS-*cu(no)* dsRNA effector.

FILE S1

Early post eclosion wing expansion phase of a *w*^{1118}*; cu*^{3}*/cu*^{4} mutant fly

File S1 is available for download as a movie file (.mov) at http://www.genetics.org/cgi/content/full/genetics.109.105601/DC1.

FILE S2

Early post eclosion wing expansion phase of a w^{1118} control fly

File S2 is available for download as a movie file (.mov) at http://www.genetics.org/cgi/content/full/genetics.109.105601/DC1.