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 more than 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 non-tissue-autonomous, systemic mode of *curled* gene function. Our study 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.

INTRODUCTION

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 upward bend, 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). Besides *Curly*, *curled* is still one of the most popular wing marker mutations represented in hundreds of fly strains used daily in Drosophila research laboratories worldwide.

Despite the widespread use of curled wing mutants the morphogenetic cause of this prominent 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 of the flies from their pupal cases (Waddington 1940). 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, there are indications for a functional interrelationship among the curled winged phenotype mutants since *curled* is incompletely dominant in *Curly* mutants (Nozawa 1956a). Additionally, the phenotypic expressivity of different curled wing mutants is variable depending on environmental factors during defined ontogenetic stages. For example, the *Curly* and *curled* mutant wing phenotypes are suppressed by larval crowding and/or undernourished larvae (Nozawa 1956a; Nozawa 1956b) a phenomenon likely to be caused by riboflavin shortage during larval stages (Pavelka and Jindrák 2001). Finally, the expressivity of the *curled* mutant wing phenotype has a cold-sensitive phase during late pupal stage (Nozawa 1956a). In summary, despite the long history of curled wing mutants which resulted in detailed genetic and phenotypic
descriptions the understanding of the gene functions and how they contribute to proper wing
morphogenesis has been hampered by the fact that none of the curled wing marker genes
has been molecularly identified to date.

Recently we performed a genome-wide 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). Subsequent analysis of various other
vertebrate tissues/organs found that all vertebrate nocturnins exert a circadian expression
mode, most prominently shown in the mouse liver (BARBOT et al. 2002; WANG et al. 2001) and
in the human hepatoma cell line Huh7 (LI et al. 2008). In nocturnin knock out mice the 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.
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 (Bönisch *et al.* 2007; Chicoine *et al.* 2007; Kadyrova *et al.* 2007; Morris *et al.* 2005; Semotok *et al.* 2005; TEMME *et al.* 2004). As yet the function of the Drosophila yCCR4-related protein subfamily representatives Angel and 3635 has not been studied. Similarly to their CCR4 relatives, the vertebrate Nocturnin proteins exert deadenylase activity *in vitro* (Baggs and Green 2003; Garbarino-Pico *et al.* 2007) and are proposed to act in posttranscriptional regulation of hitherto unidentified target transcripts. Apart form their pronounced circadian rhythm expression the vertebrate nocturnins are subject to clock-independent regulation, which implies 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). Moreover, acute regulation of mouse nocturnin by physiological cues has been proposed. In fact, nocturnin is an immediate early response gene of NIH3T3 fibroblast cells, which is characterized by its rapid induction upon phorbol ester treatment or serum stimulation and 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 with the fly ortholog of nocturnin. Disclosing 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 potential implication of fly *nocturnin* in circadian rhythm effector control and/or metabolism.

We initiated the study by showing that Drosophila *curled* (*nocturnin*) has a highly complex and dynamic ontogenetic expression pattern and that the gene is acutely regulated upon physiological challenge *in vivo*. The Curled protein features all amino acids essential for catalytic function of CCR4 proteins. It is localized in the cytoplasm, consistent with a possible function in mRNA decay via deadenylation as reported for yeast and vertebrates. We also show 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 posttranscriptional regulation of effector genes is essential for proper wing formation during the wing expansion phase early after the eclosion of flies. The *curled* mutant wing phenotype presents the first example for a morphogenetic function of a *nocturnin* gene family member, which acts in the context of the since long known Drosophila curled wing marker genes.

**MATERIALS AND METHODS**

**Fly techniques**

Flies were propagated at 25°C on a complex corn flour-soy flour-molasses medium as described (GRÖNKE *et al.* 2005).

The following fly strains were used in this study:

<table>
<thead>
<tr>
<th>Name (stock number)</th>
<th>Genotype</th>
<th>Reference/source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>tubulin</em>-GAL4 (RKF1057)</td>
<td><em>w</em>; <em>P(w^{rc}=tubP-GAL4)LL7 / TM3</em>, BDSC #5138</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
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<tr>
<td>--------------------------</td>
<td>------------------------------------------------------------------</td>
<td>------------------------------------------</td>
</tr>
<tr>
<td><strong>Df(3R)M86D (RKF1063)</strong></td>
<td><strong>P{w^{mc}=ActGFP}JMR2, Ser^1</strong> (rebalanced)</td>
<td><strong>BDSC #1714</strong></td>
</tr>
<tr>
<td><strong>FB+SNS GAL4 (RKF125)</strong></td>
<td><strong>w^*; P{w^{mW,hs}=GawB}FB+SNS</strong></td>
<td><strong>(GRÖNKE et al. 2003)</strong></td>
</tr>
<tr>
<td><strong>cu(no)</strong>^GE22476 (SGF706)**</td>
<td><strong>w^<em>; P{w^{mc}=EP}no^GE22476 renamed to w^</em>; P{w^{mc}=EP}cu^GE22476</strong></td>
<td><strong>GenExel Inc.</strong></td>
</tr>
<tr>
<td><strong>no^1 or cu^3 (SGF707)</strong></td>
<td><strong>w^<em>; no^1 renamed to w^</em>; cu^3</strong></td>
<td><strong>this study</strong></td>
</tr>
<tr>
<td><strong>no^2 or cu^4 (SGF708)</strong></td>
<td><strong>w^<em>; no^2 renamed to w^</em>; cu^4</strong></td>
<td><strong>this study</strong></td>
</tr>
<tr>
<td><strong>no^3 or cu^5 (SGF709)</strong></td>
<td><strong>w^<em>; no^3 renamed to w^</em>; cu^5</strong></td>
<td><strong>this study</strong></td>
</tr>
<tr>
<td><strong>no^GE22476rv or cu^GE22476rv (SGF710)</strong></td>
<td><strong>w^<em>; no^GE22476rv renamed to w^</em>; cu^GE22476rv</strong></td>
<td><strong>this study</strong></td>
</tr>
<tr>
<td><strong>cu(no)^{+12.2}, cu^2 (no^1) (SGF713)</strong></td>
<td><strong>w^*; P{w^{mc}=CaSpeR4}cu(no)^{+12.2}#43a/+; cu^3 (no^1)</strong></td>
<td><strong>this study</strong></td>
</tr>
<tr>
<td><strong>no(cu)^{stop12.2}, cu^3 (no^1) (RKF1067)</strong></td>
<td><strong>w^*; P{w+mC=CaSpeR4}no^{stop12.2}#20a; cu^3 (no^1)</strong></td>
<td><strong>this study</strong></td>
</tr>
<tr>
<td><strong>UAS-cu(no)-RC:EGFP (SGF811)</strong></td>
<td><strong>w^*; P{w^{mc= curled(nocturnin)} [Scer\UAS]=UAS-cu(no)-RC:EGFP}#103/ CyO float</strong></td>
<td><strong>this study</strong></td>
</tr>
<tr>
<td><strong>UAS-cu(no)-RC:EGFP; cu^3(no^1) (RKF1087)</strong></td>
<td><strong>w^*; P{w^{mc= curled=nocturnin)} [Scer\UAS]=UAS-cu(no)-RC:EGFP}#103; cu^3 (no^1)</strong></td>
<td><strong>this study</strong></td>
</tr>
</tbody>
</table>
Heat shock-induced *cu*(*no*) *in vivo* knockdown was induced by exposing the F1 progeny of the cross *hs-GAL4* x *UAS-cu*(*no*) dsRNA (raised at 25°C) for 20min to 37°C at the indicated time points/developmental stages. Equally treated F1 progeny of *w*\(^{1118}\) x *UAS-cu*(*no*) dsRNA flies were used as controls.
Generation of *nocturnin* mutants

*\textit{w}^{\text{*}}; P(\textit{w}^{+mc} = \text{EP})no(cu)^{\text{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, Inc. (Korea).

*No(cu)* deletion mutants were generated by a conventional P-element mobilization scheme resulting in the small *no3* deletion (class I event) and the two larger deletions *no1* and *no2* (class II events) as well as the precise excision allele *no^{\text{GE22476}{rev}}*, which serves as a genetically matched background control. Sequencing of the relevant part of the *no(cu)* gene showed that *no3* and *no2* deletion mutants lack genomic DNA sequences from 3R: 7.025.888 to 7.027.112 and 7.025.888 to 7.032.973, respectively, corresponding to pos. -2953 to -1729 and -2953 to +4132 relative to the putative *no(cu)* start ATG in *no(cu)*–RD exon 1. *No1* 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 (Fig. 1A).

**Fecundity assay and imaginal hatching rate**

*cu3*(*no1*) and *cu4*(*no2*) mutants were crossed into a *w^{1118}* mutant background for five generations and re-established as homozygous stocks. Embryos of the appropriate parental mutant and control genotypes (see Fig. S1A) 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-
2d after hatching for 27h. In the following single females were housed with 1-2 males each and daily transferred to new food vials. Progeny was 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.

**Molecular biology**

The following oligonucleotide primers were used in this study:

<table>
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<th>Name (stock number)</th>
<th>Sequence</th>
<th>Restriction site / comment</th>
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<tr>
<td>SGO163</td>
<td>TGCCCTGTGAGAAGTGTAGA</td>
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<tr>
<td>SGO209</td>
<td>GGGGCGTCTTAATGTATG</td>
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</tr>
<tr>
<td>SGO363</td>
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<td>BglII</td>
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<td>BglII</td>
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<td>SGO365</td>
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<td>BglII</td>
</tr>
<tr>
<td>SGO366</td>
<td>TCGGCAAAATTAGGTACCTGAAGCTTT</td>
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</tr>
<tr>
<td>SGO367</td>
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<td>Sall</td>
</tr>
<tr>
<td>SGO374</td>
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<td>SGO390</td>
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</tr>
<tr>
<td>SGO391</td>
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<tr>
<td>-----------</td>
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</tr>
<tr>
<td>SGO405</td>
<td>TGA ACTGAATCCCCCCCATCTAA</td>
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<tr>
<td>SGO406</td>
<td>GTTTTGTTGTGTTACGGAATCC</td>
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</tr>
<tr>
<td>SGO418</td>
<td>CAGT CAGTGTA CGGTC CCA</td>
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<tr>
<td>SGO419</td>
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<tr>
<td>SGO451</td>
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<tr>
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<td>Xhol</td>
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<td>SGO457</td>
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<tr>
<td>RKO692</td>
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<tr>
<td>RKO693</td>
<td>CGCTTGTTCGATCCGTAACC</td>
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</tbody>
</table>

**cDNA isolation and transgene cloning**

*cu(no)* and *SdhC* cDNAs were PCR-amplified from an embryonic (0-22h) Drosophila Oregon R cDNA library.

*cu(no)*-*RD* (pos. 75 to 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 to 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 BglII/NotI into pUAST (BRAND and PERRIMON 1993) resulting in pUAST cu\((no)\)-RC:EGFP (SG268). Using the same strategy, cu\((no)\)-RE (pos. 339 to 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 XhoI/KpnI restriction, cloned into equally restricted SG296 and the resulting 12.2 kbp genomic fragment subcloned via NotI/KpnI restriction into pCaSpeR4 vector (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\((no)\)^{stop\_12.2} 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 XbaI/KpnI 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 Fig. 2B): Amplicon a: SGO374/SGO375; amplicon b: SGO363/SGO391; amplicon c: SGO405/SGO406. Control amplicon d was amplified from the brummer gene locus using SGO163/SGO209. Bloomington Drosophila Stock Center (BDSC) fly strains #2452, #5045 (for cu1 allele) and #6591 (for cu2 allele) were crossed to transcript null no1(cu3) mutant flies. Nocturnin(curled) cDNAs of transheterozygous cu1/no1(cu3) or cu2/no1(cu3) flies were isolated as above using the primer pair SGO390/SGO366, cloned into the pCRII-TOPO vector (www.invitrogen.com) and sequenced. Cu(no) mutations detected in the cu1 allele of both cu1 mutant fly lines or in differently sized cDNAs from cu2/no1(cu3) 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; 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; GRÖNKE et al. 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 NotI linearized pCRII-TOPO cu(no)-RD (SG261), the SdhC probe of NotI linearized pCRII-TOPO SdhC (SG283) using Sp6 RNA polymerase. For quantification hybridized blots were scanned with a Phospholmager (Fujifilm BAS; www.fujifilm.com) and signal intensity was quantified using AIDA Image Analyser software v2.11 (www.raytest.de).

**In silico methods**

Insect Nocturnin (Curled) homologs from *Drosophila 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 *Drosophila melanogaster* CU-PE (ABW08641), the vertebrate Nocturnin homologs from *Xenopus 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 3rd 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 NotI 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 anaesthetized adult flies at day 4-6 post eclosion 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 post eclosion wing expansion phase of *w^{1118};cu^{3}/cu^{4}* (Movie S1) and *w^{1118}* (Movie S2) flies.

For *ex vivo* EGFP fluorescence detection fat body tissue of third instar larval progeny of the cross *Lsp-2-GAL4* x *UAS-no-RC:EGFP* was hand-dissected, embedded in phosphate-buffered saline and imaged within one hour after preparation using a Leica TCS SP2 confocal microscope using 488nm excitation and 500-541nm 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 Fig. 1A) the existence of which could be confirmed by cDNA isolation and sequencing (for details see MATERIALS AND METHODS). 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; Fig. 1A, B; see also (DUPRESSOIR *et al.* 2001)) encoded by the common last five exons. Protein sequence alignment of *Drosophila 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 (*Drosophila pseudoobscura, Anopheles gambiae*) and of non-mammalian (*Xenopus laevis*) as well as of mammalian vertebrates (*Mus musculus, Homo sapiens*) proves that NO is the bona fide Nocturnin ortholog of the fly (Fig. 1B, 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; Fig. 1B). Notably all amino acids, which have been implicated in the domains catalytic function, are completely sequence-conserved. These findings suggest that...
Drosophila NO is a putative mRNA deadenylase involved in posttranscriptional regulation of target genes (for details see Fig. 1B). Thus all three predicted Drosophila NO protein isoforms are likely to exert mRNA deadenylase function as reported for various yCCR4-related family proteins including the Xenopus Nocturnin (Baggs and Green 2003; Chen et al. 2002; 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 (Fig. 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 (for details see Materials and Methods). In addition we also recovered the precise excision revertant no$^{GE22476rv}$, which served as a genetically matched control for the subsequent experiments. No transcript expression in the deletion mutants was examined by Northern blot analysis (Fig.1D). Control flies express two prominent no transcript sizes of approximately 1.8kb and 2.0kb, referred to as S and L, respectively. According to the size of the annotated no transcripts (Fig. 1A) the S band might correspond to no-RC (1529bp) and no-RE (1856bp), whereas the L band might corresponds to no-RD (2194bp). 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 an observation supporting the specificity of the no alleles (Fig.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 bended (curled) wings and proximally crossed posterior scutellar bristles (Fig. 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**

In order to establish whether cu and no mutations affect the same gene, we performed a number of experiments which 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 (Fig. 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 (Fig. 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 (Fig. 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 (Fig. 2A,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\(^{\text{stop}12.2}\) fails to rescue the wing phenotype. This no\(^{\text{stop}12.2}\)
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 (Fig. 1A, 2A) including evolutionarily conserved and functionally important amino acid residues essential for nocturnin protein function (Fig. 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 cu²/no¹ transheterozygotes detected no gross molecular lesion of the X-ray induced cu² allele (Fig. 2B). However, genomic and cDNA sequence analysis revealed that no transcripts are indeed affected in cu¹ and cu² mutants. Cu¹ 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 (Fig. 2A,C). Similarly, cu² 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 (Fig. 2A,C). Accordingly, the NO open reading frame of the two independently generated cu mutant alleles lack the conserved endonuclease domain. Thus, cu¹ and cu² appear to be loss-of-function alleles.

Taken together, we provide ample evidence that nocturnin is identical to the historically earlier described gene curled for which reason the latter name will be maintained. Therefore, we refer in the following text to no¹, no² and no³ mutant alleles as cu³, cu⁴ and cu⁵, respectively, to the transgene bearing flies described above as cu¹212.2 and cu²stop¹2.2 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 (CURRAN *et al.* 2008; DUPRESSOIR *et al.* 1999; WANG *et al.* 2001) 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 (Fig. 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 (Fig. 3B). The proventricular and ring gland *cu* expression domains are also found at third instar larval stage (Fig. 3C). By *in situ* hybridization *cu* transcript could not be detected in the larval central brain, in the imaginal discs, the salivary glands or in the fat body (Fig. 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 (Fig. 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 (Fig. 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 does impair the fecundity of adult flies (Fig. S1A). Additionally, *cu* mutants show a normal survival rate and developmental time during ontogenesis from embryos to adult flies (Fig. S1B; 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.

Upward bend, 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]; for details see MATERIALS AND METHODS). Ubiquitous *cu* knockdown forced by different driver constructs phenocopies the *cu* mutant wing phenotype (Fig. 2G; and Table 1), whereas expression of the UAS-*cu*(no)
dsRNA effector transgene in wing imaginal discs had no impact on wing morphogenesis (Table 1, Fig. S1E). 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 1). These data suggest that the cu gene acts in a non-autonomous 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 (Fig. 2E; note the absence of wing expression shown in Fig. S1D), whereas the cu knockdown by UAS-cu(no) dsRNA effector transgene expression in the same spatio-temporal expression patterns does not interfere with normal wing development (Table 1).

In order 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 (for details see MATERIALS AND METHODS). 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 hours pre-eclosion) caused curled wings in low penetrance (<30%; Fig. 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 hours 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 about 50% (Fig. 3E). These findings show that the pupal stage is the 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; NOZAWA 1956b) 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. In order to demonstrate such a principal effect on cu mRNA, the cu transcript abundance in starved adult flies was monitored by quantitative Northern blot analysis (Fig. 3F). The results show that cu transcripts quickly accumulate upon food deprivation (2.5-fold after 6 hours) and that the gene is dynamically regulated during an extended starvation period (Fig. 3F, see also microarray data in (GRÖNKE et al. 2005); Note: That 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 post eclosion behavior?

Both aspects of the cu mutant phenotype, i.e. the posterior upward bend wings as well as the misorientation of the posterior scutellar bristle become manifest at expansion phase early after eclosion of the adult fly (Movie S1). During that stage flies behavior follows a
stereotypical though environmentally modulated complex motor pattern (Fraenkel et al. 1984). The immediate post eclosion 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 (Movie S2). This stereotyped post eclosion behavior is coordinated by the neuropeptide Bursicon (reviewed in Honegger et al. 2008), which is released into the hemolymph by a subset of NCCAP neurons during post eclosion phase II (Luan et al. 2006; Peabody et al. 2008; Peabody et al. 2009). Bursicon controls the phase III behavior but it also has somatic functions in cuticle tanning (Baker and Truman 2002; Dai et al. 2008) as well as in transient cuticle plasticization that allows body and wing expansions (Reynolds 1976; Reynolds 1977).

cu mutants show a normal phase I-III post eclosion behavior sequence suggesting that cu gene function is unlikely to be involved in the behavioral output of bursicon (burs) activity at the level of NCCAP neurons. This conclusion is supported by the finding that cu knockdown in bursicon-positive NCCAP neurons does not interfere with normal wing morphogenesis (Table 1). Moreover cu mutants tan properly (Fig. S1C and 1D). 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 post eclosion 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 Movies S1 and 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 yet. In accordance with this model we found that $cu$ does neither act 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 1).

DISCUSSION

Here we provide evidence that the Drosophila gene $cu$, initially discovered more than 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 evidences. In vertebrates as well as in Drosophila all four yCCR4-related protein subfamilies, which are termed after CCR4, nocturnin, 3635 and angel are each represented by a single ortholog of high sequence conservation (DUPRESSOIR et al.
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 (Fig. 1B; see also Dupressoir et al. 2001)). Consistent with its putative function in posttranscriptional mRNA control the EGFP-tagged CU is cytoplasmatically localized (Fig. 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 posttranscriptional control of target mRNAs via deadenylation.

Poly(A) tail removal is the first step in posttranscriptional gene repression followed by the decay of the corresponding mRNAs (reviewed in (Garbarino-Pico and Green 2007; Houseley and Tollervey 2009; Meyer et al. 2004)). 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 interaction partner of Nocturnin/Curled (Girot 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, 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 (Ceriani et al. 2002; Claridge-Chang et al. 2001; Lin et al. 2002; McDonald and Rosbash 2001; Ueda et al. 2002) nor has a subsequent metaanalysis that was based on the datasets 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 Häge 1997; Häge 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, non-circadian role for these nocturnins. In mouse and Xenopus the nocturnin genes are already expressed during early embryogenesis (Curran et al. 2008; Wang et al. 2001). In frog, nocturnin is widely expressed from neurula stages onwards starting in CNS, eye and cement gland (during neural tube stages) and in
somites, heart, pronephric tubules, otic vesicle, olfactory bulbs and pineal gland in tailbud stages. Interestingly, these 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 patterns 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); Fig. S1A, E).

A selective, modulatory role of nocturnin would be consistent with the complex though relatively subtle metabolic phenotypes of the knockout mice (GREEN et al. 2007). These mice, when kept on standard food, show reduced hepatic lipid droplet accumulation, slightly increased circulating glucose levels and moderately increased insulin sensitivity as well as glucose intolerance. On high-fat diet, however, their phenotype is more pronounced. Under these conditions they are resistant to hepatic steatosis and diet-induced obesity. Consistent with this finding several hepatic lipometabolism genes like PPAR-γ, SREBP-1c, SCD-1 and L-FABP are transcriptionally misregulated, However, they are not upregulated in the mutants, and thus their mRNAs fail to qualify as direct targets of Nocturnin. Since differences in circulating total cholesterol, triacylglyceride and free fatty acid levels are not statistically significant between mutant mice and controls it has been propose that lipid uptake from the intestine is altered in the nocturnin mutants (GREEN et al. 2007), an organ in which circadian cycling of nocturnin has not been reported. Therefore 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 aforementioned 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 (Fig. 3E) and that flies show a very sensitive phenotypic response to an acute cu downregulation (Fig. 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 portrait a gene subject to acute and dynamic regulation and thus it shows characteristics reminiscent to the non-circadian regulatory aspects of its mouse ortholog. These characteristics support a putative role for cu as an evolutionarily conserved metabolic modulator which is responsive to physiological cues. These aspects of cu function as well as the repertoire of physiological cues regulating it remain to be further elucidated.
Aside from 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 post eclosion 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 post eclosion 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? Needless to say that we have not solved the puzzle yet. However, 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 which 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 whether and how such a component could interact with the activity of the Bursicon pathway or whether it acts in parallel to it has to await the identification of first direct targets of Nocturnin proteins, may it be in vertebrates or *Drosophila*. The *Drosophila cu* mutant presents an ideal entry point for a genetic suppressor screen to possibly identify such target genes.
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FIGURE LEGENDS

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-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 grey). Residues essential for the Mg\(^{2+}\)-dependent endonuclease-like domain function are indicated as in (DUPRESSOIR et al. 2001): \(\Delta\): catalytic residue; \([\quad]\): residues involved in orientation and stabilization of catalytic residues; \(\bullet\) for phosphate binding and \(\bullet\) 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 grey) 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)*\textsuperscript{GE22476rv} controls and identifies *no\textsuperscript{1} (cu\textsuperscript{3})* as transcript null mutation. *No\textsuperscript{3} (cu\textsuperscript{5})* specifically lacks *no(cu) L* transcripts, while *no\textsuperscript{2} (cu\textsuperscript{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 upwards bend wing (left panel) and proximally crossed posterior scutellar bristle (right panel) phenotypes of *no\textsuperscript{1} (cu\textsuperscript{3})* mutants compared to *no(cu)*\textsuperscript{GE22476rv} controls.

FIGURE 2:

*curled* is *nocturnin*.

(A) Schematic overview of molecular lesions in the *cu\textsuperscript{1}* and *cu\textsuperscript{2}* alleles at the *nocturnin/curl*ed gene locus. Boxes represent non-coding (open), coding (black) or miscoding (grey) 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)*\textsuperscript{+12.2}) and with (*cu(no)*\textsuperscript{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\textsuperscript{1} (cu\textsuperscript{3})* by *Df(3R)M86D* and genomic integrity of *SdhC* in *no\textsuperscript{1} (cu\textsuperscript{3})* mutants. No gross *cu(no)* locus aberrations in the *cu\textsuperscript{2}* mutant allele. (C) *cu(no) splice* site mutation in *cu\textsuperscript{1}* and frameshift mutation in *cu\textsuperscript{2}*. The *no\textsuperscript{1} (cu\textsuperscript{3})* mutant wing phenotype is not complemented by *cu\textsuperscript{2} (D)*, can be
rescued by tissue-specific \textit{cu}(no) cDNA (E) or \textit{cu}(no)^{+12.2} genomic (F) transgene expression. (G) \textit{cu}(no) mutant phenocopy by ubiquitous expression of a \textit{cu}(no) dsRNA transgene.

\textbf{FIGURE 3:}

Complex and dynamic \textit{curled} developmental gene expression and pupal function for wing morphogenesis.

(A) Developmental Northern blot analysis detects \textit{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 \textit{cu} S transcripts. (B-C) Tissue-specific expression of \textit{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 \textit{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 \textit{curled} wing morphogenesis function in pupae determined by \textit{in vivo} RNAi. Percentage of curled winged phenotypes (black columns) and \textit{cu} transcript expression levels (grey columns) scored in flies subjected to developmental time-controlled ubiquitous \textit{cu} gene knockdown pulses during third instar larval and pupal development. Note: arrows indicate time points of heat-shock-mediated \textit{cu} knockdown induction and error bars 95% confidence intervals. (F) Quantitative Northern blot analysis demonstrates starvation-responsive transcriptional upregulation of \textit{cu} in adult male flies. Abbreviations: sg= salivary gland; pv= proventriculus; rg= ring gland; st= starved
TABLE 1:

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

Ubiquitous but no tissue-specific $cu$ knockdown causes the curled wing phenotype.

**Conditional curled knockdown causing curled wing phenotype:**

<table>
<thead>
<tr>
<th>Driver line genotype (stock source or reference)</th>
<th>Driver line tissue-specificity:</th>
<th># of F1 scored:</th>
</tr>
</thead>
<tbody>
<tr>
<td>$w^*; P(w^{+mc}=tubP-GAL4)LL7 / TM3, P(w^{+mc}=ActGFP)JMR2, Ser^1 (1m)$</td>
<td>ubiquitous</td>
<td>$&gt; 100$</td>
</tr>
<tr>
<td>$y^1 w^*; P(w^{+mc}=Act5C-GAL4)25FO1 / CyO, P(w^{+mc}=ActGFP)JMR1 (1m)$</td>
<td>ubiquitous</td>
<td>$&gt; 100$</td>
</tr>
<tr>
<td>$w^*; da-GAL4$</td>
<td>ubiquitous</td>
<td>$112^*$</td>
</tr>
</tbody>
</table>

**Conditional curled knockdown causing no curled wing phenotype:**

<table>
<thead>
<tr>
<th>Driver line genotype (stock source or reference)</th>
<th>Driver line tissue-specificity:</th>
<th># of F1 scored:</th>
</tr>
</thead>
<tbody>
<tr>
<td>$w^*; P(w^{+mw.hs}=GawB)10 (2)$</td>
<td>Wing disc</td>
<td>$133$</td>
</tr>
<tr>
<td>$w^*; P(w^{+mw.hs}=GawB)C-765 (3)$</td>
<td>Wing disc</td>
<td>$135$</td>
</tr>
<tr>
<td>$P(w^{+m}=GAL4)A9, w^* (1)$</td>
<td>Wing disc</td>
<td>$&gt;100$</td>
</tr>
<tr>
<td>$w^*; P(GAL4-vg.M2); TM2/TM6B, Tb^1 (1)$</td>
<td>Wing disc</td>
<td>$81$</td>
</tr>
<tr>
<td>$w^{1118} P(GawB-DeltaKE)Bx^{MS1096-KE}$</td>
<td>Wing disc</td>
<td>$60$</td>
</tr>
<tr>
<td>$w^*; P(w^{+mw.hs}=GawB)30A/CyO (12)$</td>
<td>Wing disc, eye-antennal disc, salivary gland</td>
<td>$37$</td>
</tr>
<tr>
<td>$y^* w^*; P(w^{+mw.hs}=GawB)FB P(w^{+m}UAS-GFP 1010T2); +/- (4)$</td>
<td>Fat body, salivary gland</td>
<td>$&gt;100$</td>
</tr>
<tr>
<td>$w^<em>; 3.1Lsp2-Gal4 line 2 / TM3, Sb^</em>$ (5)</td>
<td>Fat body</td>
<td>$54$</td>
</tr>
<tr>
<td>$y^* w^*; r^4-gal4 (6)$</td>
<td>Fat body</td>
<td>$61$</td>
</tr>
<tr>
<td>$w^*; yolk-Gal4 (II) (9)$</td>
<td>Female Fat body</td>
<td>$64$ (females)</td>
</tr>
<tr>
<td>$w^*; P(w^{+mw.hs}=GawB)FB+SNS (4)$</td>
<td>Fat body, stomatogastric nervous system, salivary gland</td>
<td>$&gt;100$</td>
</tr>
<tr>
<td>$w^{1118}; P(w^{+mc}=Sgs3-GAL4.PD)TP1 (1)$</td>
<td>Salivary gland</td>
<td>$&gt;100$</td>
</tr>
<tr>
<td>$P(w^{+mw.hs}=GawB)elav^{C155} (1)$</td>
<td>Nervous system</td>
<td>$54$</td>
</tr>
<tr>
<td>$P(w^{+mw.hs}=GAL4-Nrv2-3)$</td>
<td>Nervous system</td>
<td>$55$</td>
</tr>
<tr>
<td>$P(w^{+m}UAS-GFP)$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$w^{1118}; P(w^{+mw.hs}=GawB)drf^{PGAL8} (1)$</td>
<td>Primarily mushroom body and</td>
<td>$27$</td>
</tr>
</tbody>
</table>
**central body complexes**

- **Burs-Gal4 (8)**
- **y w*; P{w+Mc=GAL4-BS}3 (1)**
- **w*; P{w+Mw.hs=GawB}dimm (1)**
- **w*; P{w+Mw.hs=GawB}386Y (1)**
- **y w*; P{w+Mc=Ccap-GAL4.P}16 (1)**
- **P{w+Mc=GAL4-Eh.2.4}C21 (1)**

**Bursicon-positive neurons**

- **44**

**period-positive neurons**

- **65**

**Peptidergic neurons**

- **69**

**Peptidergic neurons**

- **55**

**CCAP-secreting cells of ventral ganglion and brain**

- **87**

**Eclosion hormone-expressing neurons**

- **63**

**Embryonic proventriculus, anterior midgut, posterior midgut, Malpighian tubules, and small intestine**

- **45**

**Larva: ring gland, histoblasts, gut and Malpighian tubules**

- **44**

**Adult: male accessory glands, testis sheath and cyst cells**

**Tracheal system**

- **>100**

**Muscles**

- **>100**

All driver lines were crossed against the UAS-cu(no) dsRNA effector line

* expressivity low


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**SUPPORTING INFORMATION**

**FIGURE S1.**- Fecundity, imaginal hatching rate and pigmentation of cu mutant flies and absence of pupal wing expression.

(A) Normal fecundity of cu3/cu4 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 cu3/cu4 mutant flies or females exhibiting a ubiquitous cu gene knockdown compared to the corresponding controls. (C) Comparable body pigmentation of male w1118; cu3/cu4 mutant and w1118 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.

**MOVIE 1.**- Early post eclosion wing expansion phase of a w1118; cu3/cu4 mutant fly.
MOVIE 2.- Early post eclosion wing expansion phase of a $w^{1118}$ control fly.
A

CG18577

no(cu)-RE

no(cu) dsRNA

cu¹

cu²

no(cu)stop12.2

no(cu)+12.2

B

Df(3R)M86D/ no¹(cu³)    cu²/ no¹(cu³)    wild type

M a b c d a b c d a b c d

2kbp 1kbp 0.5kbp

C

Genomic DNA:

wild type

AT CT GAAACCGAG CT CT CGGGCAGCACAAACGAT GGAT
AT CT GAAACCT CGCT CT CGGGCAGCACAAACGAT GGAT

cDNA:

wild type exon5
cu¹ exon5

CT CT CGGGCAGCACAAACGAT GGAT
CACAACCAGAT GGAT

wild type exon5
cu² exon5

AGCACCGCA A GT A CCT GATT
AGCACCGCA T T A CCT GATT

D

cu²/no¹(cu³)

E

FB+SNS GAL4 UAS-no(cu)-RC:EGFP; no¹(cu³)

F

noc(cu)+12.2 ; no¹(cu³)

G

tubulin-GAL4 UAS-no(cu) dsRNA