Supplementary Material

DENR Regulates Tissue Growth and Translation By Promoting Reinitiation Downstream of uORFs

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This file contains a Supplemental Discussion, a complete list of oligos and sequences used, and the number of replicates for each figure panel.

Supplemental Discussion

Differences between DENR function and the GCN4 paradigm

Our data reveal several important differences between the mode of action of DENR•MCT-1 and that described for the GCN4-ATF4/5 paradigms: (1) Reinitiation requires the recruitment of a new Met-tRNA$_i^{Met}$, which in all previous models including the GCN4 and ATF4/5 paradigms has been assumed to occur via *de novo* recruitment of the eIF2-containing 'ternary complex' (TC) to a 40S ribosomal subunit that has resumed scanning without an associated Met-tRNA$_i^{Met}$. A key basis for this conclusion is that efficiency of reinitiation typically increases with increasing intercistronic distance, reaching a plateau at about 80nt, presumably because the probability of TC recruitment to a reinitiating 40S subunit is increased by longer scanning time [41-45]. This length-dependence is crucial to regulation of translation of GCN4, mammalian ATF4/5, and other stress-activated genes [41,46,47]. Interestingly, we observe no such length dependence with DENR-dependent reinitiation. Indeed, DENR

promotes reinitiation equally well even at quite short intercistronic distances (compare DENR responsiveness of the 200-375 reporter (~15nt intercistronic distance) to full-length in Figure 2h). Thus, cells have a previously unappreciated means for regulating reinitiation efficiency that is fundamentally different from the TC recruitment timing mechanism that underpins regulation of yeast GCN4 and mammalian ATF4/5. (2) Regulation of GCN4 translation requires multiple cis-regulatory sequences in the GCN4 5'UTR [41]. This is not the case for regulation of mbc by DENR•MCT-1. We deleted sequences upstream of the mbc stuORFs, downstream of the stuORFs, in between the stuORFs, as well as the coding sequences of the stuORFs themselves, none of which had an effect on the ability of DENR•MCT-1 to regulate translation of the mbc reporter. In fact, simply introducing 11 nucleotides comprising a stuORF coding for one amino acid (acaaaATGTAA) is sufficient to impart DENR-dependent translation to a control 5'UTR (Figure 3a-a'), indicating that no additional cis-regulatory elements are needed. (3) The GCN4 5'UTR is a sophisticated setup that allows GCN4 translation to anti-correlate with translation of most other cellular mRNAs. Thus, when eIF2 activity levels drop and translation of most cellular mRNAs also drops, translation of GCN4 will increase, and vice-versa. Hence, translation of GCN4 is not uncoupled from general translation initiation, but rather is designed for co-regulation in an antagonistic manner. In this paradigm, the rate of reinitiation is not uncoupled from the general rate of initiation. In fact, the concomitant drop in efficiency of initiation and reinitiation due to reduced TC activity is key for antagonistically regulating GCN4 compared to other cellular mRNAs. In contrast DENR affects the efficiency of reinitiation relative to standard cap-dependent initiation, as

observed in all the luciferase reporter assays presented here, which feature normalization to a control that is translated by standard cap-dependent initiation. Hence, DENR is a molecular handle that cells can use to uncouple the rate of reinitiation from the rate of initiation.

Role of DENR in regulating cell proliferation

DENR function appears to be particularly required by cells that are proliferating. Polysome-to-monosome ratios, *de novo* protein synthesis levels, and reinitiation efficiency on stuORFs are all reduced upon DENR knockdown when cells are proliferating, but not when they are quiescent. Accordingly, the strongest developmental defects in $DENR^{KO}$ occur in histoblast nests at the onset of metamorphosis, corresponding to the moment when these cells exit quiescence and enter a phase of intense proliferation. Why proliferating cells would be more dependent on DENR is unclear at present, but one interesting possibility could be that DENR•MCT-1 function might be regulated by cellular proliferation status. Indeed, Figure 2i suggests that DENR•MCT-1 function is low by default in quiescent cells. A recent genome wide study in mouse ES cells described decreased uORF translation upon differentiation[12], which would also suggest that non-proliferating cells might have a lower requirement for reinitiation. Moreover, uORF containing transcripts are significantly enriched in vertebrate proto-oncogenes, nearly two thirds of which produce mRNAs with uORFs [48]. Drosophila stuORF transcripts also include growth-promoting kinases such as InR, PDK1 and Abl, suggesting that stuORF regulation might have a conserved function during cell proliferation.

In $DENR^{KO}$ animals, the head, thorax and limbs are not as badly affected as the abdomen (Figure 1a). Tissues forming these structures also proliferate at some point in development, so why are they not strongly affected upon DENR loss-of-function? This is likely because, compared to abdominal histoblast cells, these tissues derive from imaginal discs which proliferate earlier in development. At these earlier stages of development, maternally contributed DENR can partially rescue zygotic loss-of-function. Indeed, animals lacking both zygotic and maternal DENR (generated via ovo^{D} germline clones) die as embryos.

Consistent with DENR•MCT-1 function being modulated by cellular proliferation status, the effect of DENR loss-of-function on EcR expression also depends on whether cells are proliferating. The EcR gene has two sets of transcript isoforms both of which contain uORFs: the EcR-A isoform, expressed in tissues that are proliferating in early pupae, and the EcR-B isoform, predominantly expressed in tissues that are not proliferating. Although we could detect a clear reduction in EcR-A protein levels in $DENR^{KO}$ pupae, we could only observe a minimal reduction in EcR-B protein levels (Figure ED6c) in agreement with DENR function being mainly required in proliferating cells. This observation is supported by the fact that EcR-B levels do indeed drop upon DENR loss-of-function in S2 cells, which are proliferating (Figure 4a). This parallels the effects observed for the mbc reporter in proliferating versus quiescent cells (Figure 2i).

References for Supplemental Discussion

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Oligo sequences

For fly knockout generation:

Purpose	5' oligo	3' oligo
Amplification of the upstream flank	CCGGGCGGCCGCAACTAAATACAAAATACA	CCGGGCGGCCGCTGCGGATCGGATTAACTG
Amplification of the downstream flank	CCGGGCGGCCGCGGCGCGCCGTGAGTTCGTCTTCAAAC	CCGGGCGGCCGCGGCGCGCCCAGCTGTGGGAGCCGGCG

Oligos for dsRNA generation for S2 cell knockdowns

Purpose	5' oligo	3' oligo
lacZ	CCGGTAATACGACTCACTATAGGGACAGGGCGCGTCCCATTC	CCGGTAATACGACTCACTATAGGGGCTATGACCATGATTACGCCAAGC
GFP	CCGGTAATACGACTCACTATAGGGACCCTCGTGACCACCCTGACCTAC	CCGGTAATACGACTCACTATAGGGGACCATGTGATCGCGCTTCTCGT
DENR	CCGGTAATACGACTCACTATAGGGAGGCGTCACCTATCCGATCCAGATGA	CCGGTAATACGACTCACTATAGGGAGGCGGGTATGACATCGAACAAGTCA
MCT-1	CCGGTAATACGACTCACTATAGGGAGGCCACATCGAACTGCTGCTAA	CCGGTAATACGACTCACTATAGGGAGGTGGATAACGTGAGGAGTCCA
Ligatin/eIF2D	TAATACGACTCACTATAGGGGCTCCTGTGACCTTCGCTAC	TAATACGACTCACTATAGGGTAAAGTTCACCACTTCGGGG
MCT1 3'UTR	GGCCTAATACGACTCACTATAGGGCGAAATAGGAATCTGCACTTGC	GGCCTAATACGACTCACTATAGGACCGCCAAACAGAAGACTCAATG
CG10498	GGGGGGCACCCAAATGTGGTCCAAC	GGGGGGCGATGCAGGATACGATTC

Oligos for mbc luciferase reporter cloning

Purpose	Used in Figure:	5' oligo	3' oligo
mbc promoter + 5'UTR	ED2b	CCGGAGATCTGAATGTGTGGAAAATGTATT	CCGGTTCGAAGTCATCTTGAAGGCTTTTCTTCT
mbc full-length 5'UTR	2E-I,3A'-C, ED2E-H	CCGGCTGCAGAGTACGGTACTTCTGGGC	CCGGTTCGAAGTCATCTTGAAGGCTTTTCTTCT
mbc 5'UTR 100-575	ED2e	CCGGCTGCAGCACACACGAGTGTGT	CCGGTTCGAAGTCATCTTGAAGGCTTTTCTTCT
mbc 5'UTR 200-575	ED2e	CCGGCTGCAGGCGAAAAACGGCGCA	CCGGTTCGAAGTCATCTTGAAGGCTTTTCTTCT
mbc 5'UTR 300-575	ED2e	CCGGCTGCAGGCTTTTCGTCGAAAT	CCGGTTCGAAGTCATCTTGAAGGCTTTTCTTCT
mbc 5'UTR	ED2e	CCGGCTGCAGCGTTGCCTAATTTAA	CCGGTTCGAAGTCATCTTGAAGGCTTTTCTTCT

400-575			
mbc 5'UTR 500-575	ED2E	CCGGCTGCAGAAGTCGGCGCAAAAA	CCGGTTCGAAGTCATCTTGAAGGCTTTTCTTCT
mbc 5'UTR 233-425	ED2G	CCGGCTGCAGACAATTTGTGCGGAA	CCGGTTCGAAGTCATCTTCAACAAGACATTAAATTA
mbc 5'UTR 266-425	ED2G	CCGGCTGCAGTAATTATTTATTGGTT	CCGGTTCGAAGTCATCTTCAACAAGACATTAAATTA
mbc 5'UTR 200-500	ED2F	CCGGCTGCAGGCGAAAAACGGCGCA	CCGGTTCGAAGTCATCTTTTACCACCAACACGGCGA
mbc 5'UTR 200-425	ED2G	CCGGCTGCAGGCGAAAAACGGCGCA	CCGGTTCGAAGTCATCTTCAACAAGACATTAAATTA
mbc 5'UTR 200-400	ED2G	CCGGCTGCAGGCGAAAAACGGCGCA	CCGGCTGCAGCGTTGCCTAATTTAA
mbc 5'UTR 200-375	2H, ED2G	CCGGCTGCAGGCGAAAAACGGCGCA	CCGGTTCGAAGTCATCTTTTTTGATTTCGTTTTCAC
mbc 5'UTR 200-350	ED2F	CCGGCTGCAGGCGAAAAACGGCGCA	CCGGTTCGAAGTCATCTTCCAACACAAGCATTTCGC
mbc 5'UTR 200-275	ED2F	CCGGCTGCAGGCGAAAAACGGCGCA	CCGGTTCGAAGTCATCTTTAAATAATTATTGCACAA
mbc 5'UTR ATG mutation T219A	2E	AAACGGCGCAAAAAGCATTTGCGGAG	CTCCGCAAATGCTTTTTGCGCCGTTT
mbc 5'UTR ATG mutation A249G	2E	AATTTGTGCGGAGTGCTAGCTTTTGT	ACAAAAGCTAGCACTCCGCACAAATT
mbc 5'UTR ATG mutation A338T	2E	AATCGGAGGCGAATTGCTTGTGTTGG	CCAACACAAGCAATTCGCCTCCGATT
mbc 5'UTR Kozak mutation 218	2F	GAGCGAAAAACGGCGGTGTATGCATTTGCGGAGC	GCTCCGCAAATGCATACACCGCCGTTTTTCGCTC
mbc 5'UTR Kozak mutation 248	2F	AGCGACAATTTGTGGTGTATGCTAGCTTTTGT	ACAAAAGCTAGCATACACCACAAATTGTCGCT
mbc 5'UTR Kozak mutation 338	2F	AGCGAAATCGGAGGGTGTATGCTTGTGTTGGC	GCCAACACAAGCATACACCCTCCGATTTCGCT
mbc 5'UTR CDS swap uORF 249	2G	TTTGTGCGGAATGCAACAACAACAACAATAATTATTTAT	ATAAATAATTATTGTTGTTGTTGTTGCATTCCGCACAAA
mbc 5'UTR CDS swap uORF 339	2G	CGGAGGCGAAATGCAACAACAACAACAACAATGAAAACGAAATC	GATTTCGTTTTCATTGTTGTTGTTGTTGTTGCATTTCGCCTCCG
mbc 5'UTR CDS swap uORF 219 (into already mutated uORF249 construct)	2G	ACGGCGCAAAATGCAACAACAACAACAACAACAACAACAATGCAACAACAAC	GTTGTTGTTGCATTTGTTGTTGTTGTTGTTGTTGTTGCATTTTGCGCCGT
mbc 5'UTR	2H	AGCTTTTGTGCAATTATTAT	ACCAATAAATAATAATTGCACAAA

stop codon mutation 268		TTATTGGT	AGCT
mbc 5'UTR stop codon mutation 360	2H	CCGGCTGCAGGCGAAAAACG GCGCA	CCGGTTCGAAGTCATCTTTTTTGA TTTCGTTTTCCCAATGCC
mbc 5'UTR remove sequence between uORFs	"data not shown"	CTAGCTTTTGTGCAATAAGA CGTCGAAATGCTTGTGTTG	CAACACAAGCATTTCGACGTCTTA TTGCACAAAAGCTAG

Oligos for 5'UTR luciferase reporter cloning

Purpose	**Used in Figure:**	**5' oligo**	**3' oligo**
EcR-RB	3B	CCGGCTGCAGATTTAGTATTT TTTTG	CCGGTTCGAAGTCATCTTCCTCTG GCAGCCGATA
gfa	3B	CCGGCTGCAGAGTTGCAGTTG GAAAGTC	CCGGTTCGAAGTCATCTTTATTCT ATATCCTATATT
TyrR	3B	CCGGCTGCAGCGCCGGCTTAC CTGATCGA	CCGGTTCGAAGTCATCTTGGATCG GCCAGTTGGGTTCA
CG3558	3B	CCGGCTGCAGGCTGCTCATCG CTAACTTTC	CCGGTTCGAAGTCATCTTTTGTCG GTGCGGTGTGTGTG
snoo-RB	3B	CCGGCTGCAGTGCAAAAATAA TTTGCA	CCGGATCGATGTCATCTTTTTTGG GCAATACTTT
InR-RA	3B	CCGGCTGCAGGGCTTGTCCGG GTGT	CCGGTTCGAAGTCATCTTATTGCG GTGTTTCTA
janB	3C	CCGGCTGCAGTAGCTTTGCAA CTGCTCG	CCGGTTCGAAGTCATCTTTTTATT TAACCAAAGGAA
CG8963	3C	CCGGCTGCAGAAATAATAAAT TTTAACA	CCGGTTCGAAGTCATCTTGTTTTC GAGTCCAACGCA
psh	3C	CCGGCTGCAGCGGTCAGAACC GTCGTCC	CCGGTTCGAAGTCATCTTCTTAAT TCGCTTCGACAC
drm	3C	CCGGCTGCAGAGTTATTAGTA GCATTCA	CCGGTTCGAAGTCATCTTTTTGAC AGGCGGTATGTG
Rbp6-RC	3C	CCGGCTGCAGCCTTCAGTCGT TATCAGA	CCGGATCGATGTCATCTTAACGAG CTTCTTTGTTGG
CG43674	3C	CCGGCTGCAGCATTTCTGTTA GAGTGCT	CCGGTTCGAAGTCATCTTGGCTGG CTAAAGCGCCTT

Oligos for generating plasmids for making reporter mRNAs by in vitro transcription reactions

Purpose	**Used in Figure:**	**5' oligo**	**3' oligo**
actin 5'UTR into RLuc (control)	ED2 C-D	GGCCAGATCTTAATACGACTCA CTATAGGCATATCACTACCGTT TGAGTTC	GGCCTTCGAAGTCATTTTGTAAGA TTTGGTGTGTTTT
actin 5'UTR into FLuc (normalization control)	ED2 C-D	GGCCAGATCTTAATACGACTCA CTATAGGCATATCACTACCGTT TGAGTTC	GGCCCCATGGTTTGTAAGATTTGG TGTGTTTT
mbc 5'UTR	ED2 C-D	CCGGAGATCTTAATACGACTCA CTATAGGAGTACGGTACTTCTG	CCGGTTCGAAGTCATCTTGAAGGC TTTTCTTCT

Oligos for introducing synthetic uORFs into a control 5'UTR

Purpose	Used in Figure	5' oligo	3' oligo
mutagenizing CG43674 5'UTR to introduce SpeI and AgeI sites	3A-A', ED5A, ED10E, ED10F-G	TCTGTTAAAGAAAAACTAGTGTGACCGGTAATTAAAATAATTACTAG	CTAGTAATTATTTTAATTACCGGTCACACTAGTTTTTCTTTAACAGA
1 aa	3A-A', ED5A, ED10E, ED10F-G	CTAGTGTGTCCGGACAAAATGTAAGCCGCCGCCGCCGCCGCCGCCA	CCGGTGGCGGCGGCGGCGGCGGCGGCGGCTTACATTTTGTCCGGACACA
4 aa	3A-A'	CTAGTGTGTCCGGACAAAATGGCCGCCGCCTAAGCCGCCGCCGCCGCCA	CCGGTGGCGGCGGCGGCGGCTTAGGCGGCGGCCATTTTGTCCGGACACA
9 aa	3A-A'	CTAGTGTGTCCGGACAAAATGGCCGCCGCCGCCGCCGCCGCCTAAA	CCGGTTTAGGCGGCGGCGGCGGCGGCGGCTTACATTTTGTCCGGACACA

Oligos for inducible stuORF and control luciferase reporter cloning

Purpose	Used in Figure	5' oligo	3' oligo
Fluc ORF	ED7	ACCGAATTCGAGATGGAAGACGCCAAAAAC	AACCTCGAGTTACACGGCGATCTTTCC
Subcloning of the control and 1 aa uORF synthetic constructs	ED7	CCGGAATTCCATTTCTGTTAGAGTGCT	TCATAAACTTTCGAAGTC

Oligos for generating the plasmid for the inducible MCT-1 stable cell line

Purpose	Used in Figure	5' oligo	3' oligo
MCT-1 ORF	ED10c	CACGGTACCGAATTCACCATGGCGTTCAAAAAATTCGAAGAGAA	CACGCGGCCGCTTACTCGAGCTTCACGGGCTTCGACTTCC

Oligos for quantitative RT-PCR

Gene	Used in Figure	5' oligo	3' oligo
rp49 (norm. control)	2B',4C, ED1F,ED2D-D',ED6A-C', ED7G'	GCTAAGCTGTCGCACAAA	TCCGGTGGGCAGCATGTG
mbc	2B-C, ED2D',ED10A'	CCGCAACTCTGTTGAAACC	CGGGGTCCAATCCATGTT

InR	ED6A',ED6C'	TCCCCAGCGAAAAATTAAG	GACTATGTCGGCAAATTTCTT
EcR	ED6C'	GGACCAGATCACGTTACTAAAGGC	GCAGGTCTTCAATGTTATCAGCC
Br-C	4C	AGGAGATCGGCGACGGAC	TTGAGACCTAGCAACGCTGAG
E75	ED6B	GCAGCAGCAGATCGGAATACTC	CCGACTCAATGCCCGAATCC
DENR	ED1B,1F	CACGATGCCCATTGAGTAC	CGGCAGCCTCCTCCTCAA
RLUC	ED2D'	GGGTGCTTGTTTGGCATTTC	GGCCATTCATCCCATGATTC
MCT1	ED7G'	GAGGCCTATCCCAAGTTG	CGATGTGGTCATGGCACT
18S rRNA	ED5D-D'	CGGAGAGGGAGCCTGAGAA	AGCTGGGAGTGGGTAATTTACG
28S rRNA	ED5D-D'	GAAATCCGCTAAGGAGTGTGTAACA	CAACTTAAGCGCCATCCATTTT
Initiator tRNA	ED4E, ED5D-D'	AGCAGAGTGGCGCAGTGGAAGC	TAGCAGAGCAAGGTTTCGATCCTCGG

Oligos for RT-PCR with crosslinked RNA

Gene	**Used in figure**	**5'oligo**	**3'Oligo**
Rps13	ED4E	CCCTCCAAAATCGGCATC	GCGACGGCCTTCTTGATC
Cdc2	ED4E	ATCAACCGCGATCAGAGA	GGCAGCGAATCCATGTAT
InR	ED4E	TCCCCAGCGAAAAATTAAG	GACTATGTCGGCAAATTTCTT
RPL32	ED4E	ATGACCATCCGCCCAGCATACA	TGCGCTTGTTCGATCCGTAACa
mbc	ED4E	GGATGGAAAGGTTAAGGAG	CGTCTTGGGACCACTTAGA
Initiator tRNA	ED4E	AGCAGAGTGGCGCAGTGGAAGC	TAGCAGAGCAAGGTTTCGATCCTCGG

Oligos for reporter fly lines

	Used in figure	5'Oligo	3'oligo
CG43674 (control and stuORF reporter)	ED8 and ED9	CCGGGGTACCCATTTCTGTTAGAGTGCT	CCGGGGATCCGGCTGGCTAAAGCGCCTTT
RFP	ED9	CCGGGGATCCACCGGTCGCCACCATGGCCTCCTCCGAGGAC	CCGGGCGGCCGCTTAGGCGCCGGTGGAGT

Oligos for Rescue experiments with MCT1 Luciferase assay

	Used in Experiment	**5'Oligo**	**3'Oligo**
MCT1	ED10E	CCGGGGTACCATGTTCAAAAAATTCGAA	CCGGGCGGCCGCCTACTTCACGGGCTTCGA
T82A	ED10E	GGCCCCTGGATGCCTGCCCTGCGCCTCCTGC	GCAGGAGGCGCAGGGCAGGCATCCAGGGGCC
TS118/119AA	ED10E	ATGTGTCCCGGCCTCGCCGCCCCAGGCGCCTGTATG	CATACAGGCGCCTGGGGCGGCGAGGCCGGGACACAT
T125A	ED10E	CCAGGCGCCTGTATGGCCCCGGCCGACAAGGAC	GTCCTTGTCGGCCGGGGCCATACAGGCGCCTGG
T118A	ED10E	ATGTGTCCCGGCCTCGCCTCGCCAGGCGCCTGTATG	CATACAGGCGCCTGGCGAGGCGAGGCCGGGACACAT
S119A	ED10E	ATGTGTCCCGGCCTCACAGCCCCAGGCGCCTGTATG	CATACAGGCGCCTGGGGCTGTGAGGCCGGGACACAT

Number of Replicates for figure data

Figure	Number of replicates (n=)
1d	3
1e	3
1f	5
1g	4
1h	3-4
1i	4
1i'	4
2a	3

2b’	3
2c	Already on the figure
2e	3
2f	3
2g	3
2h	3
2i	3
3a’	3
3b	3
3c	3
4c	3
4d	20-40
4e	Already on the figure
ED1c	3
ED1e	10-20
ED1g	3
ED1o	3
ED1q	4
ED1r	4
ED2b	3
ED2c	3
ED2d	3
ED2d’	3
ED2e	3

ED2f	3
ED2g	3
ED2h	3
ED3c	10
ED4g	3
ED5a	3
ED5d	Already on the figure
ED5d’	Already on the figure
ED6a	3
ED6a’	3
ED6b	3
ED6c’	3
ED6d	20-40
ED7c	3
ED7d	On the figure
ED7e	On the figure
ED7f	3
ED7f’	3
ED7h’	3
ED10d	4
ED10e	3
ED10f	3