**Cell Reports, Volume *17***

## Supplemental Information

# PDF Signaling Is an Integral Part of the *Drosophila* Circadian Molecular Oscillator

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**Supplemental legends:**

**Figure S1: Related to Figure 1. The *tim*Tomato reporter construct responds to CLK activity in S2 cells and manifests circadian-like oscillations *in-vivo.* A.** Schematic representation of the cloning strategy used to generate the *tim*Tomato reporter construct. Briefly, a cassette containing pCaSpeR4 MCS flanking the *Drosophila* codon optimized Tomato-NLS-PEST CDS with SV40 3'-UTR was inserted into pCaSpeR4 MCS between XhoI and KpnI sites (Red). This resulted in the reconstitution of the MCS and allowed subsequent insertion of *Timeless* promoter and 5'-UTR between XhoI and AvrII sites (Red and Green). Other restriction sites flank the different transcribed regions (i.e. Fluorophore, NLS, PEST and 3'-UTR) thus allowing modularity of these features. **B.** CLK expression in S2 cells activates transcription from the *tim*Tomato reporter. Top: comparison between features of the *tim*Tomato and *tim*YFP constructs. Bottom: Representative pictures of S2 cells transfected with CLK expressed under constitutive (pActin-CLK) or inducible metallothionine promoter (1mM $Cu^{2+}$ pMT-CLK). The low TOMATO signal relative to YFP signal (per cell) is possibly due to the high turnover rate of the PEST. Signal intensity in control wells (No treatment and 0 uM $Cu^{+2}$) demonstrate the specificity of the 6.4 kb *tim* promoter relative to the leaky 0.7 kb promoter. **C.** Western blot (WB) analysis showing *in-vivo* oscillations of TOMATO in transgenic fly heads. 8 transgenic lines were generated using random insertion of the transgene to the genome. The lines were screened by WB analysis for oscillations in TOMATO, TIM and VRI. At least five lines (i.e. 3, 5, 6, 7, 8) display oscillations in TOMATO signal.

**Figure S2: Related to Figure 1. The *tim*Tomato reporter manifests oscillations in transcription in the circadian neurons with phase and amplitude similar to the *timeless* gene products. A.** The *tim*Tomato reporter (Red) allows visualization of all neuronal subgroups in the circadian system with high specificity, and does not require immunostaining. **B.** TOMATO signal (red) is co-localized with signal from anti-PDF immunostaining (green) to the *pdf*-expressing cells. **C.** Real-time PCR shows mRNA levels of *tim* and *vri* normalized to *tubulin* across the day in *tim*Tomato fly heads. Error bars represent standard deviation (S.D) of three biological repeats. **D.** A representative picture of a gel from WB analysis that shows the levels of TIM, VRI and TUBULIN in *tim*Tomato fly heads throughout the day from three biological repeats. **E.** Neuronal oscillations in TOMATO. The signal of endogenous TOMATO (red) and GFP (green) in the LNds (Right) and DNs (Left) of UAS-mcd8GFP; *tim*Tomato/*pdf*GAL4 fly brains collected and dissected at the indicated time. Flies were entrained for 3

days in 12:12 Light:Dark (LD) conditions, collected and dissected at the indicated time-points. **F**. Oscillations in TOMATO in brains of reporter flies kept for 3 days under free running conditions (DD3). Top: Representative pictures of brains of *tim*Tomato flies that were dissected at the indicated time in DD3. Bottom: Quantification of the TOMATO signal from the whole brain. Error bars represent S.D of three biological repeats. **G.** Activation of CLKGR in circadian neurons by dexamethasone (Dex) induces TOMATO expression. Representative picture of transgenic *tim*GAL4/*tim*Tomato;UAS-CLKGR (*tim*CLKGR-*tim*Tomato) brains. Flies were grown on vehicle (45% cyclodextrin, Left) or Dex (2.5 mM, Right) containing food under 12:12 h light:dark (LD) conditions for 96h. Brains were dissected at ZT19 and visualized for TOMATO signal (red)**. H.** Representative picture of TOMATO signal in *tim*Tomato/ClkW.T (Left) and *tim*Tomato/CLKSV40 brain (Right), dissected at ZT7. **I.** Oscillations of VRI in the LNds and DNs of cultured brains. Whole mount immunohistochemistry performed on dissected Cantonese S. (CS) brains post-culture. Brains were incubated under 12:12h LD conditions for four days and then collected and stained for VRI (Green) to assess oscillations during the 5$^{th}$ day in culture.

**Figure S3: Related to Figure 2. The *tim*Tomato reporter follows dynamics in CLK-driven transcription in ex-vivo culture in real-time and at a single cell resolution.** *tim*GAL4/*tim*Tomato; UAS-CLKGR fly brains (*tim*CLKGR-*tim*Tomato) show constant elevation in TOMATO signal in all circadian neuronal groups. Cultured brains were stimulated with Dex and immediately imaged. Left: Single cell quantification of the response and the average signal per group (Error bars represent S.D of the single cell measurements). Right: Snap shots of starting (zero h) and end (96h) point positions of representative brains. Single cell ROIs determined for the quantification of TOMATO signal during the time-lapse imaging (1 frame/30min) are shown in colored circle. **A.** LNvs **B.** LNds **C.** DN1s and DN3s.

**Figure S4: Related to Figure 2. Activation of CLK transcriptional activity in the LNvs promotes down regulation in CLK-driven transcription in the rest of the network A.** Frequency plots of the number of TOMATO positive cells per neuronal subgroup of cultured *pdf* CLKGR-*tim*Tomato brains (Based on the same samples and genotype indicated in Figure 2A). Statistical significant was determined for the difference in distributions between vehicle and Dex-treated brains using two-sample Kolmogorov-Smirnov test (K-S test). NS=not significant, * $p< 0.05$ ** $p<0.01$, *** $p<0.001$ ****

p<0.0001. **B.** Dexamethasone (Dex) was applied on control *tim*Tomato;UAS-CLKGR (CLKGR-*tim*Tomato) cultured brains. The kinetic of the response was monitored by time-elapsed imaging (1 frame / 40min). The quantification shows that this does not stimulates any change in TOMATO signal in the different neuronal groups indicated. **C.** Comparison of the quantification of TOMATO signal based on the number of cell detected in the same samples of brains indicated in Figures 2A and 2B, and that were cultured under vehicle conditions only.

**Figure S5: Related to Figure 3. CLK regulates *pdf* expression**. **A.** *Ex-vivo* activation of CLK-dependent transcription by Dex in the LNvs of *pdf*GAL4/*tim*Tomato;UAS-CLKGR (*pdf*CLKGR), leads to increased PDF levels at ZT19 as measured by immune-staining (Green). Left: Representative pictures of vehicle (Top) and Dex (Bottom) treated brains. Right: Quantifications of the response using intensity (Top) and cell number count (Bottom). $N_{Dex}$=39, $N_{vehicle}$=37 hemispheres. **B.** Expression of *Clk* RNAi reduces PDF expression. Brains were immune-labeled with anti-PDF (Purple) at ZT5. Top: representative pictures of *pdf*Tomato/$CLK^{RNAi}$ brains ($CLK^{RNAi}$) (Left) and *pdf*GAL4;*pdf*Tomato/$CLK^{RNAi}$ (*pdf*GAL4;$CLK^{RNAi}$) (Right). Bottom: quantification of PDF expression in the LNvs of the same samples analyzed in Figure 3B. N=24, 25 hemispheres respectively. Statistical significance was determined using two tail Student's T-test, NS=not significant * p< 0.05 ** p<0.01, *** p<0.001 **** p<0.0001. Error bars represent SEM. **C.** Left: Scheme of the *pdf*Tomato transcriptional reporter. Sizes of the different modules are written above. Restriction sites are indicated below the scheme. NLS: Nuclear Localization Signal; PEST: mouse ornithine decarboxylase; SV40-3'-UTR: Simian Virus 3' Untranslated Region. Right: Representative picture demonstrating the specificity of the reporter.

**Figure S6: Related to Figure 4 and Table 1. Identification of putative regulators of *pdf* using Yeast One Hybrid (Y1H).** Schematic representation of the fragments of the *pdf* control region that were generated in order to identify putative regulators using a yeast one hybrid (Y1H) screen. The screen identified interaction with 27 genes (Table 1). Interestingly, 4 of these genes are CLK targets and enriched in the LNvs, and they interact with *pdf* most proximal promoter fragment generated. Two of these genes are also Mef2 direct targets - Hr38 and SR.

**Figure S7: Related to Figure 4. Neuronal Activity and the activity regulated genes - *Hr38* and *sr* - regulate *pdf* expression. (A-C)** *Pdf*GAL4 and *tim*GAL4 flies containing a UAS-dicer2 transgene (*pdf*GAL4, dcr2) were crossed *dhr38* and *sr* RNAi lines. PDF immuno-labeling (green) was performed at ZT5 on brains carrying different genotypes as indicated in the figure. Shown are representative pictures (Top) and quantifications (Bottom). **A.** Expression of $hr38^{RNAi}$ transgene in the *pdf*-expressing cells causes large reduction in PDF levels (N=27, 22, 30 hemispheres respectively). **B.** Expression of $hr38^{RNAi}$ transgene in clock cells using the *tim*GAL4 driver causes large reduction in PDF levels (N=27, 22, 23 hemispheres respectively). Control brains of UAS-$HR38^{RNAi}$ are common to the experiments presented in A and B that were performed together). **C.** Expression of $sr^{RNAi}$ transgene in the *pdf*-expressing cells causes large increase in PDF levels (N=24, 32, 30 hemispheres respectively. **D.** *pdfGAL4;pdf*Tomato/UAS-TrpA1 and *pdf*Tomato/UAS-TrpA1 flies were incubated at $33^0$C for 2h. TOMATO (red) and PDF (green). (Based on the same samples and genotype indicated in Figure 4C). Statistical significance was determined using two tail Student's T-test, NS=not significant * $p< 0.05$ ** $p<0.01$, *** $p<0.001$ **** $p<0.0001$. Error bars represent SEM.

**Movie S1: Related to Figure 2.** Following dynamics in CLK-driven transcription in the LNvs at a single cell resolution using the *tim*Tomato circadian transcriptional reporter.

**Movie S2: Related to Figure 2.** Following dynamics in CLK-driven transcription in the LNds at a single cell resolution using the *tim*Tomato circadian transcriptional reporter.

**Movie S3: Related to Figure 2.** Following dynamics in CLK-driven transcription in the DNs at a single cell resolution using the *tim*Tomato circadian transcriptional reporter.

**Movie S4: Related to Figure 2.** Down-regulation in transcription from the *tim*Tomato in the DNs in response to activation of CLK-driven transcription in the LNvs.

**Supplemental Experimental Procedures:**

**Cloning of reporter constructs and DNA baits of the *pdf* promoter:**

To generate the *tim*Tomato reporter construct, a reconstitution cassette (for the pCaSpeR4 MCS) containing a *Drosophila* codon optimized TdTomato-NLSx3-PEST coding sequence with a SV40 3'-UTR (synthesized by GenScript) was cut from pUC57 carrier vector and inserted into pCaSpeR4 between XhoI-KpnI sites (See Figure S1A for details about restriction sites included in the cassette). A previously characterized 6.4 kb fragment containing the promoter and 5'-UTR stretching into to the second exon ATG in the *timeless* gene (Stanewsky et al., 2002) was amplified from genomic DNA using the forward primer (include a XhoI restriction site) 5'-TCCGAACTCGAGTCCAGGTCAACACTGTCATA-'3 and reverse primer (includes a AvrII restriction site) 5'-ACGGTTCCTAGGCGACTGCGAACATTGAGGTA-'3, and ligated between XhoI and AvrII sites in the reconstructed MCS of pCaSpeR4.

To generate the *pdf*Tomato reporter construct a previously characterized (Park et al., 2000) 2.45 kb fragment containing the genomic region upstream to the *pdf* gene transcription start site, was cut from a carrier vector and ligated in a pattB-sv40 based vector between BamHI and EcoRI. *Drosophila* codon optimized TdTomato-NLSx3-PEST coding sequence with a SV40 3'-UTR was cut from pUC57 carrier vector and inserted downstream to the *pdf* promoter in the pattB-sv40 vector between EcoRI and NotI.

To generate DNA baits of the *pdf* promoter for *Drosophila* transcription factor screen (Y1H) we used a carrier vector containing the 2.45 kb promoter of the *pdf* gene as a template to generate five overlapping PCR fragments approximately 600 bp long, that contain restriction sites for BamHI-Acc65I, using the following sets of primers:

For: CGCGGATCCTCCGTGGGTTTCATCCTTAC Rev: CGGGGTACCAGGAGCGTCTTGGTCACATC

For: CGCGGATCCACGAATCATCTTCGGCTTGT Rev: CGGGGTACCCAGTCACACAACGCACATCA

For: CGCGGATCCTGTGGCTGCATGGAAAGTTA Rev: CGGGGTACCCCTCCTCCTCCTCCTCCTC

For: CGCGGATCCTTGGAACCTAGCCCTGATTG Rev: CGGGGTACCATAGCTAGCTCGGCAGTTGG

For: CGCGGATCCCAAGACAATTGGCGGAATTT Rev: CGGGGTACCAGCAGGAGACTTGCGAAT GA

Fragments were cloned into BamHI-KpnI site in a pENTRY-5' vector and then sub-cloned using the recombination based Gateway cloning strategy into pMW2 pBD-HIS using LR Clonase (Invitrogen).

***Drosophila* adult brain culture:**

**Post-culture applications:** Visualization of TOMATO or immune-staining of the brains were performed immediately after fixation to avoid fading of TOMATO signal. For time-course experiments, fixed brains were re-immersed in 0.2% PFA and placed in 4˚C until all samples collected (more than 72h in 4˚C under these conditions will result in high TOMATO background signal in the sample).

**Immunofluorescence:**

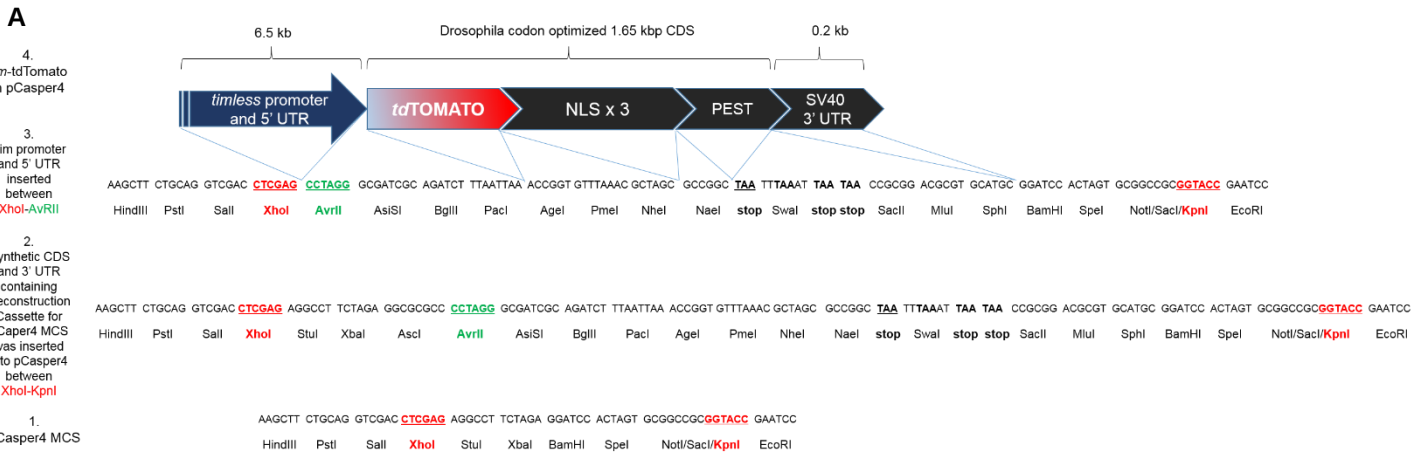
Performed as previously described (Lerner et al., 2015) with minor modifications. 1st antibodies were used at: 1:1500 dilution for mouse Anti-PDF (a gift from the Justin Blau lab), 1:1000 Rat anti-TIM (a gift from Michael Rosbash) and 1:2000 guinea pig anti-VRI (a gift from Paul Hardin). The Anti-mouse, anti-rat and anti-guinea pig 2nd antibodies were diluted 1:1000 (Alexa Fluor conjugated 488, 555 and 633).

**qPCR:**

Real-time RT-PCR was performed as described in (Weiss et al., 2014), using the same primer sequences for tim and vri. Primers for Drosophila codon optimized Td-Tomato used in this study were: For: 5'-TGGACATCACGTCGCATAAT-'3 and Rev: 5'-TACAGCTCATCCATGCCGTA-'3.

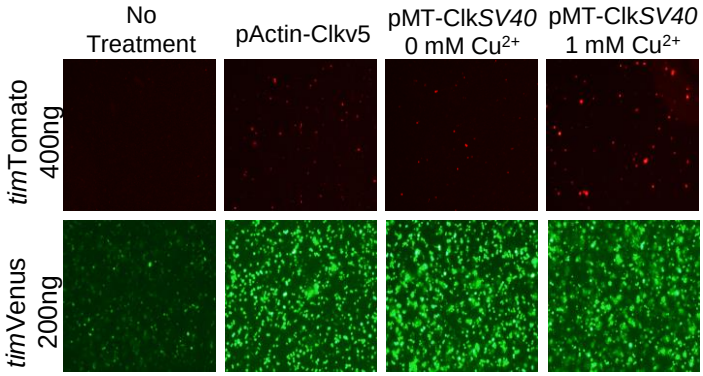
**Western Blot analysis:**

Western blots with anti-VRI, anti-TIM and anti-TUBULIN antibodies were performed as described in (Weiss et al., 2014). TOMATO was detected by anti-mouse DsRed polyclonal antibody (purchased from Clontech).




B

| Reporter | Promoter and construct (Kbp) | CDS (Kbp) | Other Features |
|---|---|---|---|
| *Tim*-tdTomato | 7 promoter (17 vector) | 2.0 Tandom dimer | optimized PEST + NLS |
| *Tim*-Venus | 0.7 promoter (9 vector) | 0.7 Monomer | |

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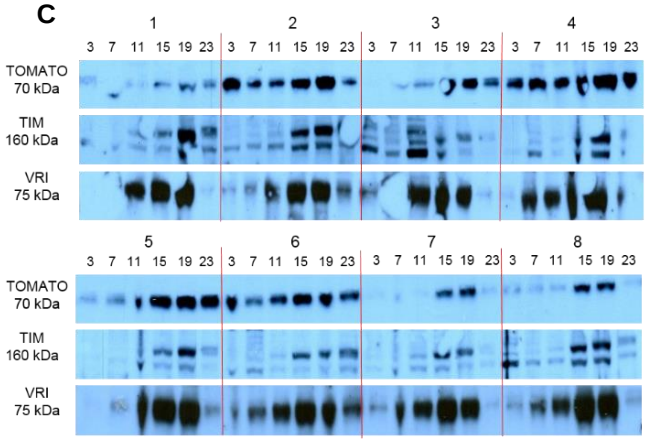
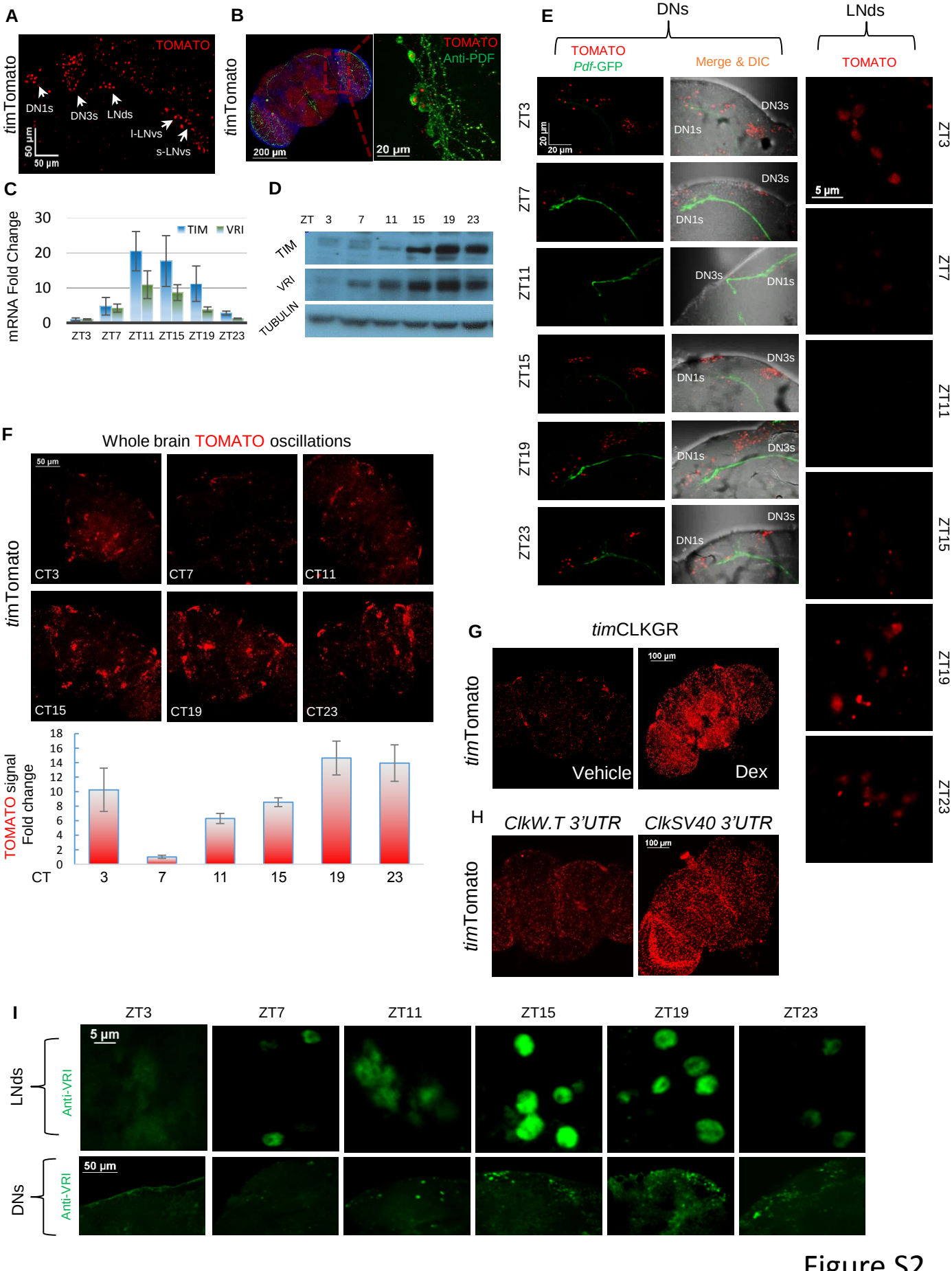



Figure S1


A
timTomato
TOMATO
DN1s
DN3s
LNds
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Anti-PDF
200 µm
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C
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30
20
10
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VRI
ZT3 ZT7 ZT11 ZT15 ZT19 ZT23
D
ZT 3 7 11 15 19 23
TIM
VRI
TUBULIN
E
DNs
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TOMATO
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Merge & DIC
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timTomato
50 µm
CT3
CT7
CT11
CT15
CT19
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TOMATO signal
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timTomato
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Figure S2

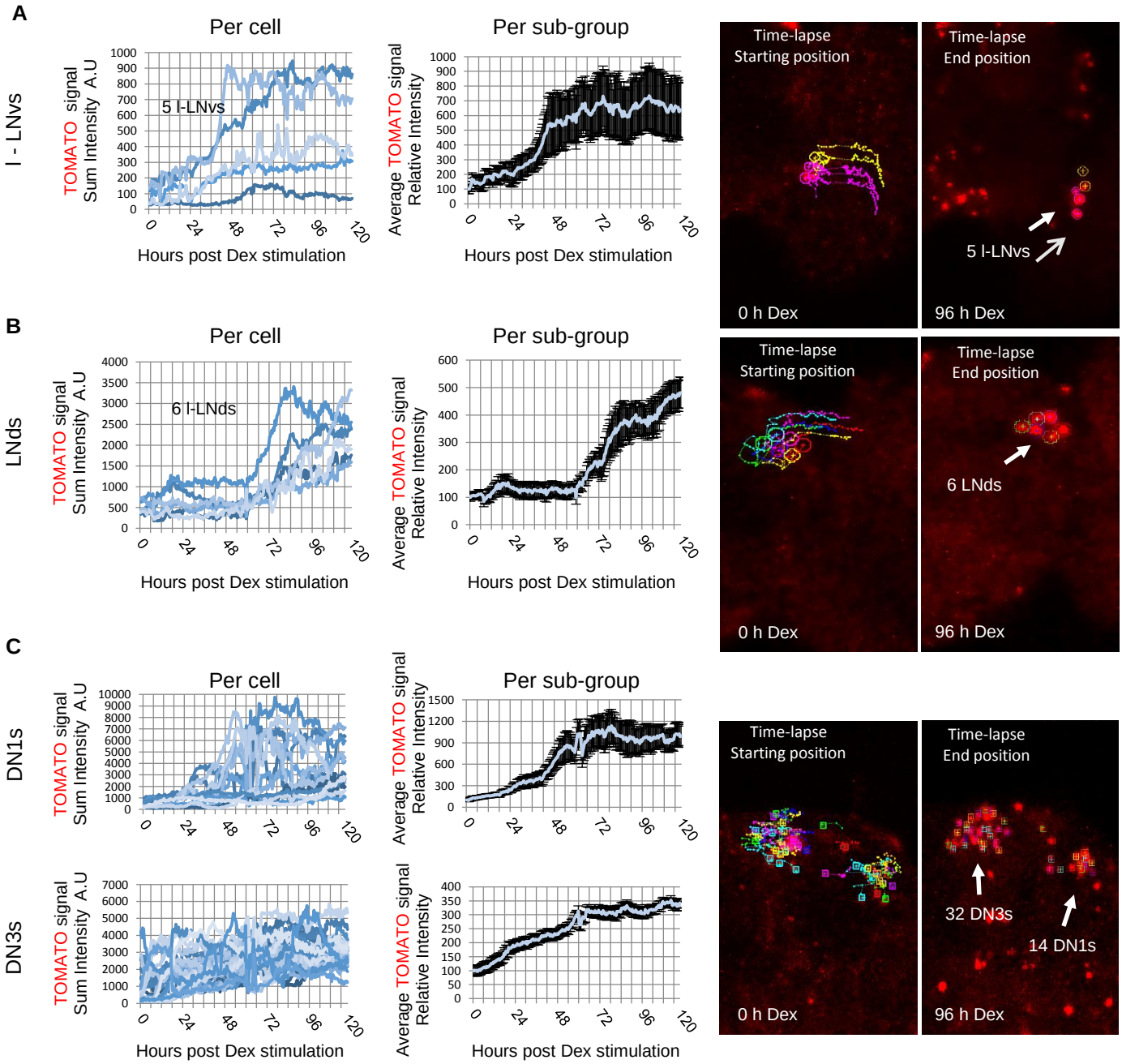
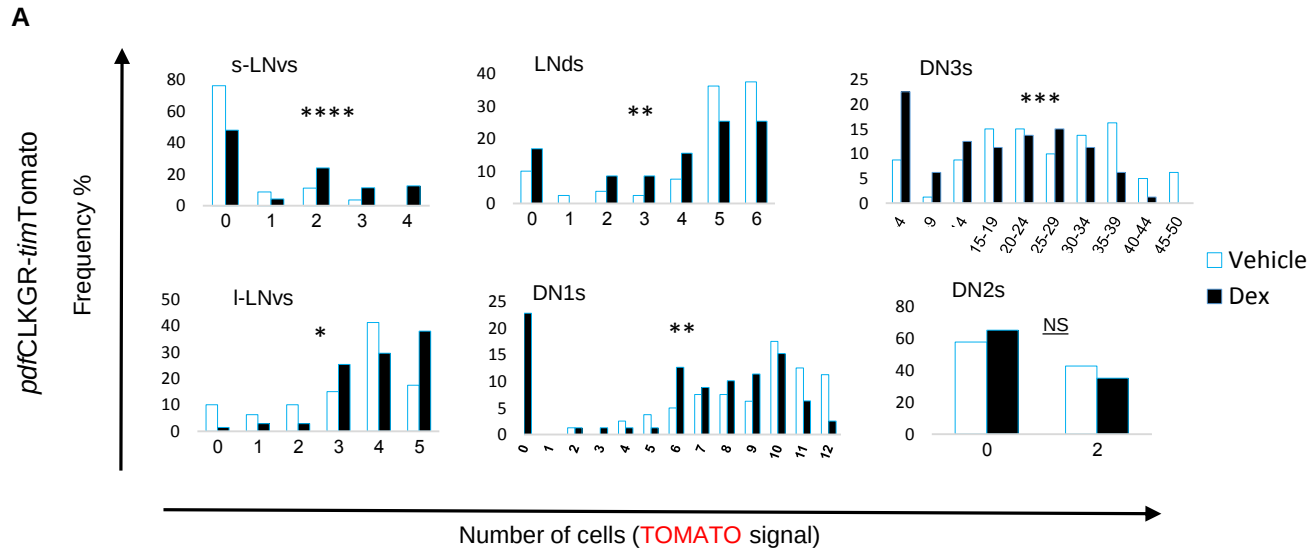

A
l - LNvs
Per cell
TOMATO signal
Sum Intensity A.U
5 l-LNvs
Hours post Dex stimulation
Per sub-group
Average TOMATO signal
Relative Intensity
Hours post Dex stimulation
Time-lapse
Starting position
0 h Dex
Time-lapse
End position
5 l-LNvs
96 h Dex
B
LNds
Per cell
TOMATO signal
Sum Intensity A.U
6 l-LNds
Hours post Dex stimulation
Per sub-group
Average TOMATO signal
Relative Intensity
Hours post Dex stimulation
Time-lapse
Starting position
0 h Dex
Time-lapse
End position
6 LNds
96 h Dex
C
DN1s
Per cell
TOMATO signal
Sum Intensity A.U
Per sub-group
Average TOMATO signal
Relative Intensity
Time-lapse
Starting position
0 h Dex
Time-lapse
End position
32 DN3s
14 DN1s
96 h Dex
DN3s
TOMATO signal
Sum Intensity A.U
Hours post Dex stimulation
Average TOMATO signal
Relative Intensity
Hours post Dex stimulation

Figure S3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Frequency %
s-LNvs
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DN1s
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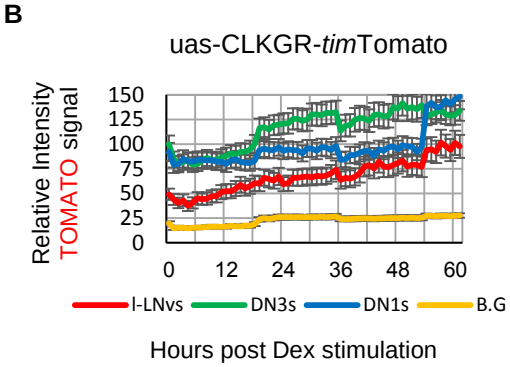
DN2s
NS
Vehicle
Dex
Number of cells (TOMATO signal)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uas-CLKGR-timTomato
Relative Intensity
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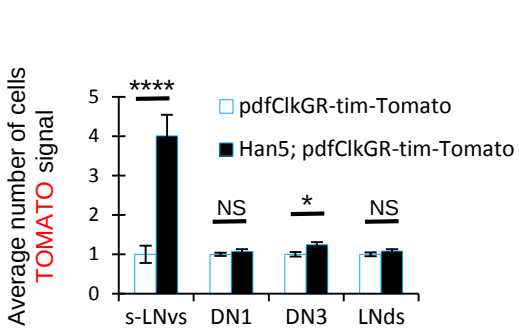
DN3s
DN1s
B.G
Hours post Dex stimulation


C
Average number of cells
TOMATO signal
****
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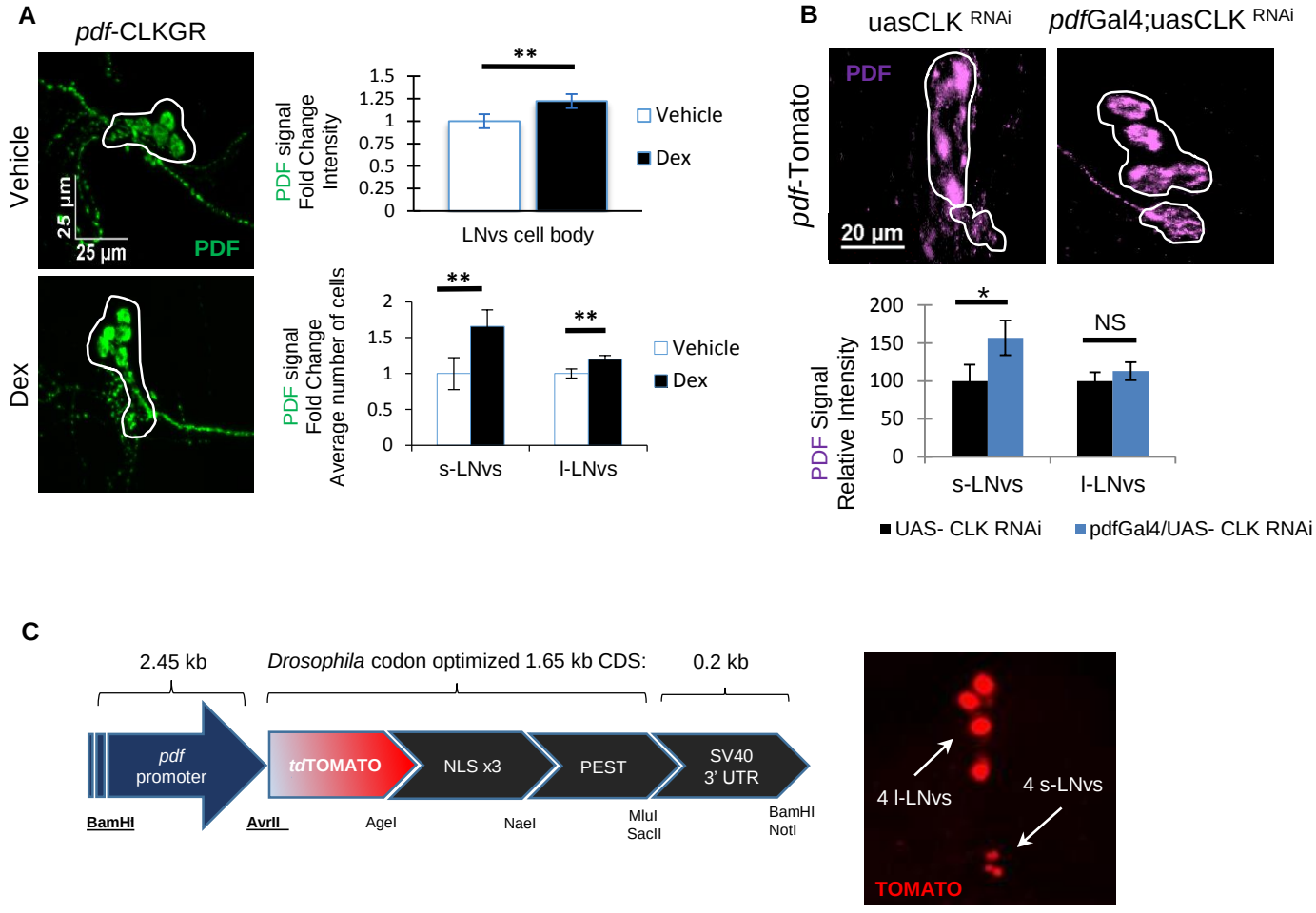
Han5; pdfClkGR-tim-Tomato
s-LNvs
DN1
DN3
LNds

Figure S4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SV40 3' UTR
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NotI
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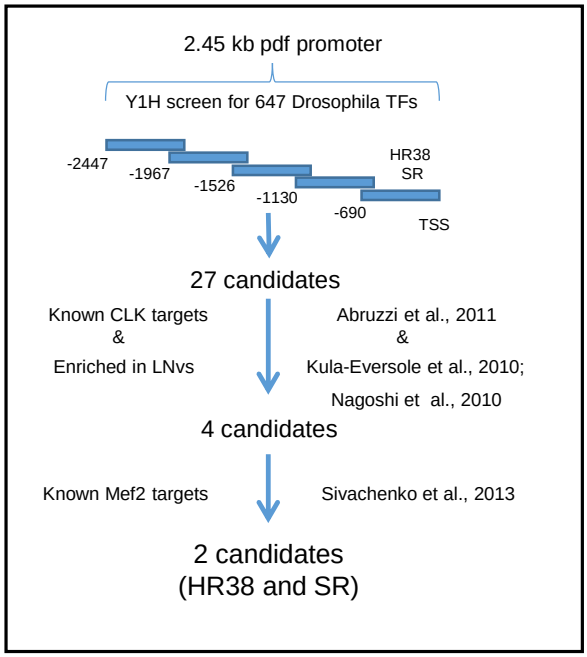
4 s-LNvs
TOMATO

Figure S5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HR38
SR
TSS
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Known CLK targets
&
Enriched in LNvs
Abruzzi et al., 2011
&
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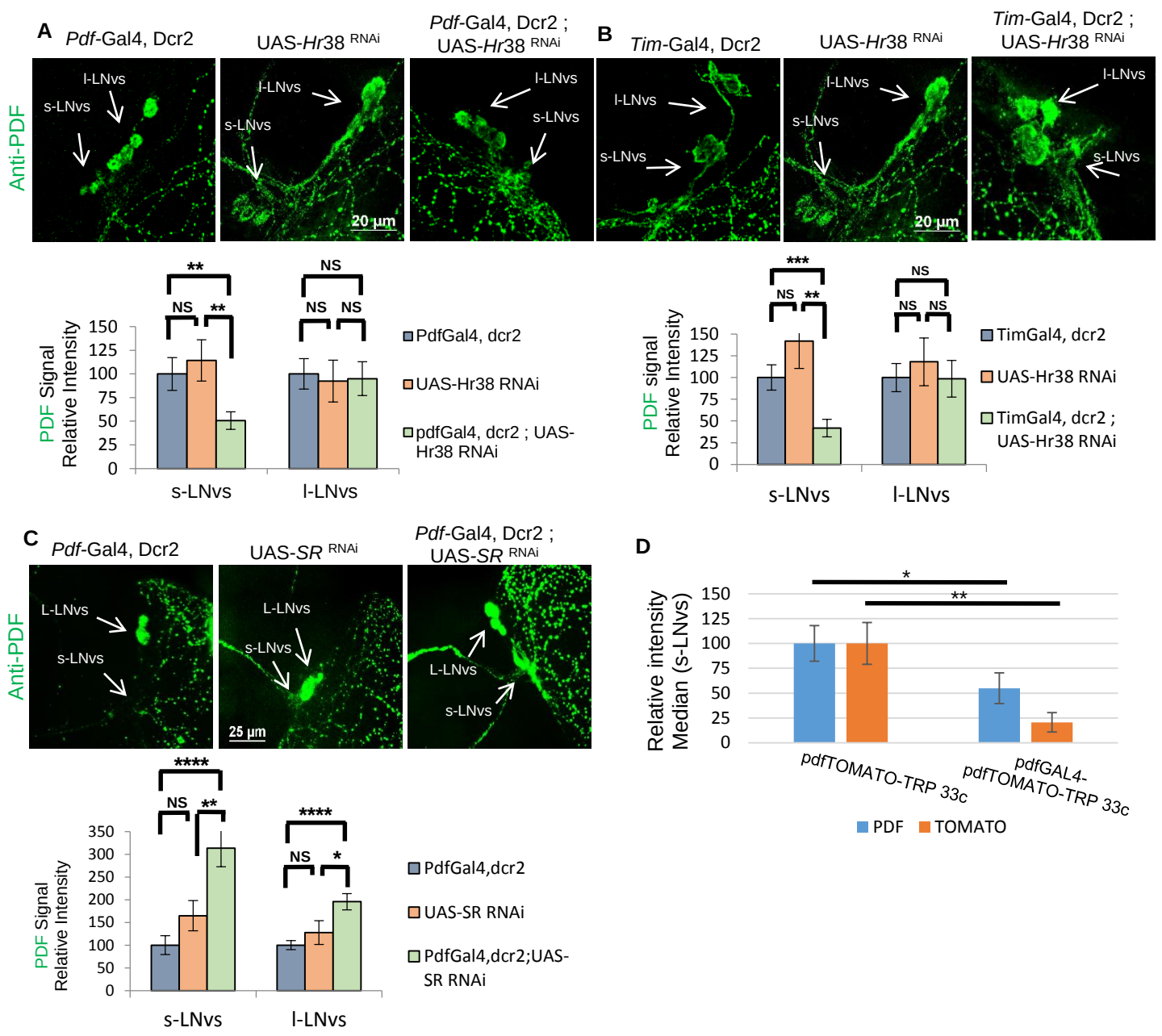
2 candidates
(HR38 and SR)

Figure S6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B
Tim-Gal4, Dcr2
UAS-Hr38 RNAi
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PDF Signal Relative Intensity
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Figure S7