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Single base pair differences in a shared motif determine differential *Rhodopsin* expression

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Abstract

The final identity and functional properties of a neuron are specified by terminal differentiation genes, which are controlled by specific motifs in compact regulatory regions. To determine how these sequences integrate inputs from transcription factors that specify cell types, we compared the regulatory mechanism of *Drosophila Rhodopsin* genes that are expressed in subsets of photoreceptors to that of phototransduction genes that are expressed broadly, in all photoreceptors. Both sets of genes share an 11bp activator motif. Broadly expressed genes contain a palindromic version that mediates expression in all photoreceptors. In contrast, each *Rhodopsin* exhibits unique single bp substitutions that break the symmetry of the palindrome and generate activator or repressor motifs critical for restricting expression to photoreceptor subsets. Novel sensory neuron subtypes can therefore evolve through single base pair changes in short regulatory motifs, allowing the discrimination of a wide spectrum of stimuli.

In the visual system, different photoreceptor neurons express specific light-sensing pigments (1); however, common downstream factors amplify and convert the response to the visual stimulus into a neuronal signal. For instance, each unit eye (ommatidium) of the *Drosophila* retina contains eight photoreceptors (R1-R8) that express different light-sensing Rhodopsins (Rhs) that are restricted to specific photoreceptor subsets. Outer photoreceptors R1-R6 express Rh1. Inner photoreceptors R7/R8 express either Rh3 in **p**R7s coupled with Rh5 in **p**R8s, or Rh4 in **y**R7s with Rh6 in **y**R8s (Fig. 1A) (1). R1-R8 all share broadly expressed phototransduction factors (Fig. 1B and fig. S1A) that amplify and convert the response to the visual stimulus into a neuronal signal (2).

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SUPPLEMENTARY MATERIALS

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Supplementary Text

Materials and Methods

figs. S1–S9

Supplementary References

Here, we examine the *cis*-regulatory mechanisms that distinguish restricted from broad expression patterns for Rhodopsins and downstream phototransduction factors, respectively. All *Rhs* share the conserved *Rhodopsin* Core Sequence I/RCSI (3, 4), which resembles the palindromic P3 motif (TAATYNRATTA), an optimal binding site for Paired-class homeodomain proteins (5). Almost all known broadly expressed phototransduction genes contain a P3 motif in their proximal promoter (Fig. 1B, fig. S1A, and Supplementary Text). The presence of a conserved P3/RCSI motif within 100bps of the *Rh* transcription start site (TSS) is significantly associated with enrichment in adult eyes (χ-squared test, p-value < 0.001). P3/RCSI is required for activation in photoreceptors since its mutation caused either a loss or a strong reduction in expression of 16 broad or restricted reporters (figs. S1, S2 and S3), with the exception of *Arr1* (fig. S2K). Moreover, expression of 10 out of 15 reporters was lost in mutants for the photoreceptor-specific transcription factor Pph13 (Fig. 1B, figs. S2 and S3), a Paired-class homeodomain protein that binds P3 and the *Rh6* RCSI *in vitro* (6, 7).

Since each *Rh* promoter has a highly conserved RCSI variant (Fig. 1B) (4), we tested the sufficiency of P3 and RCSI to determine the significance of the specific differences between perfectly palindromic (P3) and imperfect motifs (RCSI) (Fig. 2). Four copies of the P3 motif (including four neighboring bps for spacing; the contribution of these additional bps was only tested for *Rh4*, see below) from the broadly expressed *ninaC, rdgA,* or *trpl*, drove broad expression in all photoreceptors (Fig. 2, A and A', and fig. S4, A and A'), consistent with our previous results (8). In sharp contrast, multimerized RCSI motifs drove expression in subsets of photoreceptors. The RCSI of *Rh3* and *Rh6* contains a K_{50} motif, a binding site for K_{50} homeodomain proteins such as the Dve repressor or the Otd activator (Fig. 1B). Expression of **[*Rh3 RCSI*]$_4$** and **[*Rh6 RCSI*]$_4$** was biased to inner photoreceptors: **[*Rh3 RCSI*]$_4$** mediated restricted expression in R8 and in R7, with a strong bias towards the **p**R7 subset where *Rh3* is normally expressed (Fig. 2, B and B'). This pattern is complementary to the expression of Dve (Fig. 1B) (9), which is indeed responsible for the restricted expression as [*Rh3 RCSI*]$_4$ drove a broad, P3-like pattern in *dve* mutants (Fig. 2 B''). **[*Rh6 RCSI*]$_4$** drove restricted expression in R8s and R7s; expression in R1-R6 was very weak in comparison to P3 motifs, which was due to *dve*-dependent repression (fig. S4D, D' and D'').

[*Rh1 RCSI*]$_4$ drove variable expression in R1-R6 (Fig. 2, C and C'), where *Rh1* is expressed. This outer photoreceptor-specific pattern is complementary to the inner photoreceptor expression of **[*Rh3 RCSI*]$_4$**. *Rh4* has the same RCSI as *Rh1*. However, adding the synergistic 3' RCSII motif (fig. S1, F–I) (3) led to expression in **y**R7s, where *Rh4* is expressed (fig. S4, B and B', and fig. S4C and C'). Although **[*Rh5 RCSI*]$_4$** was not sufficient for reporter expression (fig. S4, E and E'), adding three K_{50} motifs to a single *Rh5* RCSI (**[*K$_{50}$*]$_3$+[*Rh5 RCSI*]$_1$)** led to expression in R8 and **p**R7 photoreceptors (fig. S4, F and F').

In summary, the RCSI motifs of specific *Rhs* differ from palindromic P3 motifs in broadly expressed genes: They drive expression that is biased towards the endogenous *Rh* expression patterns (Fig. 2D). We show below that full subtype-specificity and activation often requires the repetition of motifs that are present in the RCSI.

As specific RCSI motifs directed restricted expression in different photoreceptor subsets (Fig. 2D), albeit with incomplete subtype-specificity and with some variability in expression levels, we asked whether the single bp differences are required for subtype-specificity in a wild type promoter context, and which other motifs are required for full restriction. We mutated the K_{50} (Otd/Dve) motifs (TAAT**CC**) to Q_{50} (Pph13) motifs (TAAT**TG/A**) (Fig. 1B) to disrupt repression while preserving RCSI-mediated activation. Mutating the ***Rh3* RCSI** resulted in an expansion to **y**R7s where Dve is present at low levels (Fig. 3, A and B). Mutating the ***Rh6* RCSI** caused de-repression in R1-R6 and the ocelli (fig. S5, A and B, and fig. S6A). *Rh3* and *Rh6* have K_{50}/Dve repressor motifs repeated upstream and mutation of individual motifs also caused de-repression in **y**R7s (Fig. 3C) (10) and R1-R6/ocelli (fig. S5D and fig. S6B), respectively. Taken together, single bp changes create K_{50} motifs in the *Rh3* and *Rh6* RCSI, which are required for subtype-specific expression together with their upstream repeats.

We also examined the significance of the disrupted P3 palindrome, i.e. the imperfect 3' homeodomain binding motif in the RCSIs of *Rh1-Rh5* (Fig. 1B). Creating a palindromic motif in the ***Rh3* RCSI** (TAATCCAATT**C**→TAATCCAATT**A**) caused de-repression in **y**R7s (Fig. 3D) that depended on *Pph13* (Fig. 3E). Therefore, de-repression appears to be due to increased activation through the newly created Q_{50}/Pph13 site. The same ATT**C**→ATT**A** mutation in the ***Rh5* RCSI** led to partial de-repression in **y**R8s (fig. S5, E and F). This single bp change created a binding site for the activator Otd (AGATTA) (11) and indeed de-repression in **y**R8s was lost in *otd* mutants, as was activation in **p**R8s (fig. S5G).

The 3' ATTC motif in the RCSI of *Rh3* and *Rh5* is repeated upstream. Mutating the upstream repeat without creating a Q_{50}/Pph13 site (ATTC→CAAA) also caused de-repression in **y**R7s (*Rh3*) or **y**R8s (*Rh5*) (Fig. 3F and fig. S5H). Mutating both ATTCs of *Rh5* enhanced de-repression into almost all **y**R8s (fig. S5I). Therefore, we have identified repressor motifs in the RCSIs of four *Rhs* (K_{50}/Dve motifs in *Rh3*/*Rh6* and ATTC motifs in *Rh3*/*Rh5*). These motifs are repeated upstream within less than 100 bps and are required for full subtype-specificity.

A single bp ATT**T**→ATT**A** mutation in the ***Rh4* RCSI** caused de-repression in R1-R6, **p**R7s and the ocelli (Fig. 3, G and H, and fig. S6D). The correct pattern was restored by crossing the mutant *Rh4* reporter in a *Pph13* mutant background (Fig. 3I), indicating that the A→T change prevents Pph13 from overcoming repression in the 'wrong' photoreceptor subsets, as was the case for *Rh3* and *Rh6*. The same mutation in the ***Rh1* RCSI** caused no detectable de-repression (fig. S5J). Replacing two bps in the RCSI of the ocelli-specific ***Rh2*** (fig. S6, E and F) to obtain a Q_{50}/Pph13 site led to de-repression in R1-R6 photoreceptors that depended on *Pph13* (fig. S6, G and H).

Our *in vivo* data reveal that a cell-fate decision requires single bp differences in RCSI motifs (Fig. 3J). They complement previous findings in cell culture that subtle sequence differences in a glucocorticoid receptor or NF-κB binding site can specify the mode of transcriptional regulation (12, 13), and that small differences in binding site sequences can lead to distinct Hox specificities *in vivo* and *in vitro* (14–16). *(i)* Single bps in RCSI prevent binding of

dimers of broadly expressed activators such as Pph13 (5) (fig. S8B), tipping the balance of activator/repressor binding. This weakened activation allows repressors to prevent activation in other photoreceptor subtypes (17). *(ii)* They generate specific combinations of overlapping activator and repressor motifs, often repeated upstream to provide robust expression and full subtype-specificity. Creating overlap of activator and repressor motifs is an efficient way of blocking a key activator site in the 'wrong' cell types that express a repressor (18), especially since the RCSI motifs are very close to the transcription start site and repression there could block other activators (19). The precise tuning of RCSI motifs within their respective promoter context leads to incompatibility in other *Rh* promoters, as revealed by RCSI swap experiments: Replacing a given RCSI with another one resulted in two main outcomes, loss of expression or de-repression in specific subsets of photoreceptors (fig. S7 and Supplementary Text).

The RCSI/P3 motif resembles 'terminal selector' motifs that allow the coordinated expression of effector genes that define a particular neuron type (20, 21). Yet, RCSI motifs exhibit additional layers of regulation that are integrated in a single regulatory element, as their sequence is modified for subtype-specificity. Mutating a *cis*-regulatory motif in many cases appears to be the shortest evolutionary path towards a novel phenotype (22). Although we found that it is possible in some cases to eliminate ectopic expression by removing the broadly expressed activator Pph13 (Fig. 3, E and I), this simultaneously causes a loss of expression of several broad phototransduction genes, defects in photoreceptor morphology and a severe loss of light sensitivity (23).

We propose that the modification of a P3-type motif into different RCSI-type motifs allowed partitioning *Rh* expression to different subtypes of photoreceptors (Fig. 4 and fig. S8). This opened the possibility to discriminate wavelengths and likely conveyed a selective advantage. In this model, P3 motifs represent a positive regulatory element shared by ancestral genes that were expressed in all photoreceptors. This regulation is conserved, as the promoter of the long wavelength Rh as well as *Gβ76C* that are both expressed in all photoreceptors in the beetle *Tribolium*, contains a palindromic P3-type motif and depend on Pph13 (24, 25).

In conclusion, our study revealed a high level of precision at every base pair in a short *cis*-regulatory element that is critical for proper spatial (broad or restricted) expression. It will be interesting to see whether similar modifications of shared *cis*-regulatory motifs are used to diversify neuronal cell types in other developmental contexts, for instance in human photoreceptor and olfactory genes (fig. S9, Supplementary Text and Tables).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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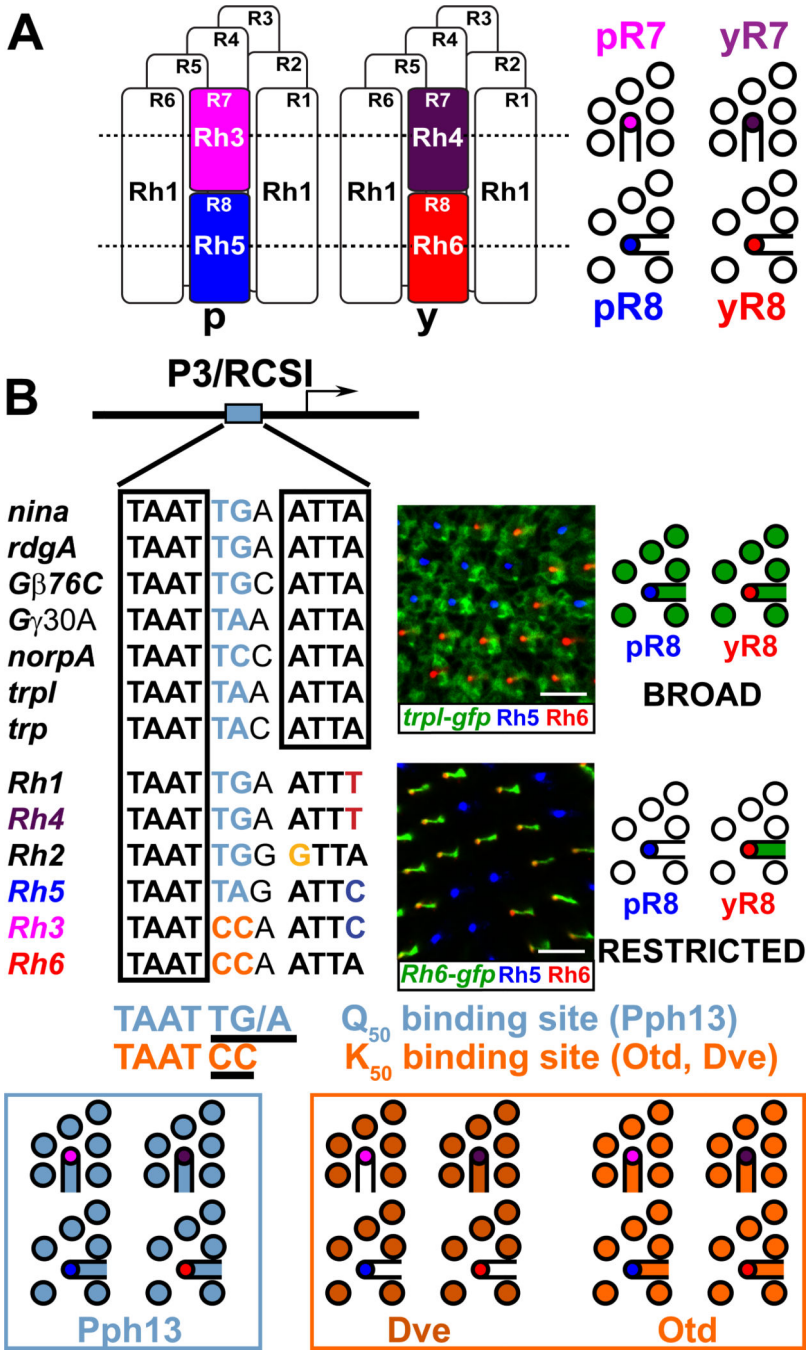


Fig. 1. Broadly expressed and restricted photoreceptor genes share a *cis*-regulatory motif

(A) In **p** ommatidia, Rh3 in **p**R7 is coupled with Rh5 in **p**R8, whereas in **y** ommatidia, Rh4 in **y**R7 is coupled with Rh6 in **y**R8. Outer photoreceptors R1-R6 express Rh1. **Right**: Cross-section at the level of R7 (top) or R8 (bottom).

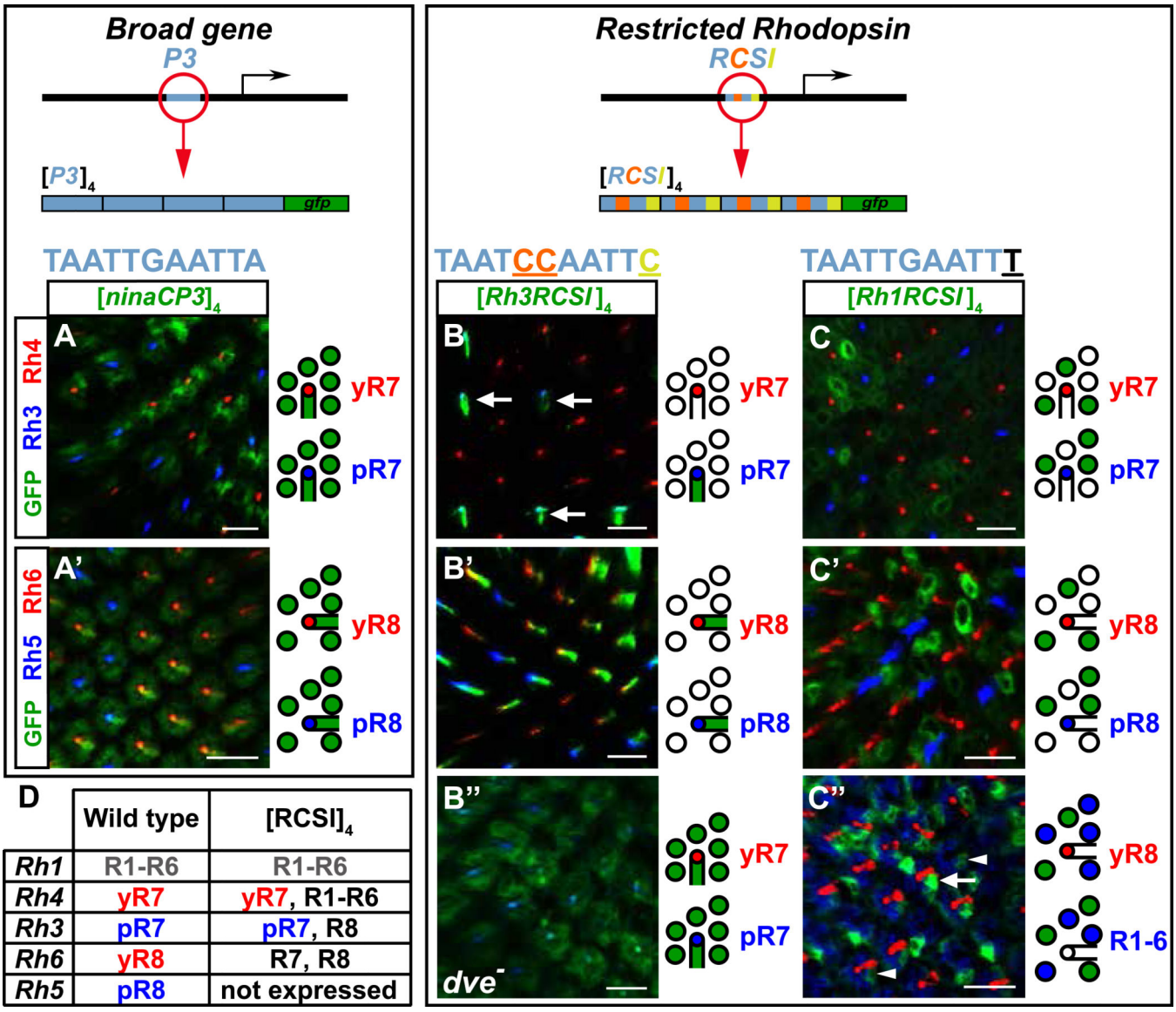
(B) Broadly expressed photoreceptor genes (7 upper genes) and restricted *Rhodopsins Rh1-Rh6* share the 11 base pair P3/RCSI motif in their proximal promoters.

Left: All motifs contain a 5' TAAT homeodomain core binding site, which is repeated in reverse orientation (ATTA) in broadly expressed phototransduction genes. The 3' ATTA is

modified in RCSI motifs of *Rh1-Rh5*. In *Rh6* and *Rh3*, central bp differences (orange) create K_{50} sites (TAATCC) for the activator Otd and the repressor Dve (9). Q_{50} sites (TAATTG/A) are bound by the photoreceptor-specific activator Pph13 (6).

Right: Reporter expression patterns of the broadly expressed phototransduction gene *trpl* and the restricted *Rh6* at the R8 level. Retinas were stained for GFP (green), Rh5 (blue) and Rh6 (red). Scale bars, 10 μm.

Bottom: Pph13 and Otd are expressed in all photoreceptors, whereas Dve is expressed at high levels in R1-R6 and at low levels in **y**R7s.



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	Wild type	[RCSI]4
Rh1	R1-R6	R1-R6
Rh4	yR7	yR7, R1-R6
Rh3	pR7	pR7, R8
Rh6	yR8	R7, R8
Rh5	pR8	not expressed

Fig. 2. The P3 motif is sufficient to drive expression in all photoreceptors, while RCSI motifs drive expression in subsets of photoreceptors

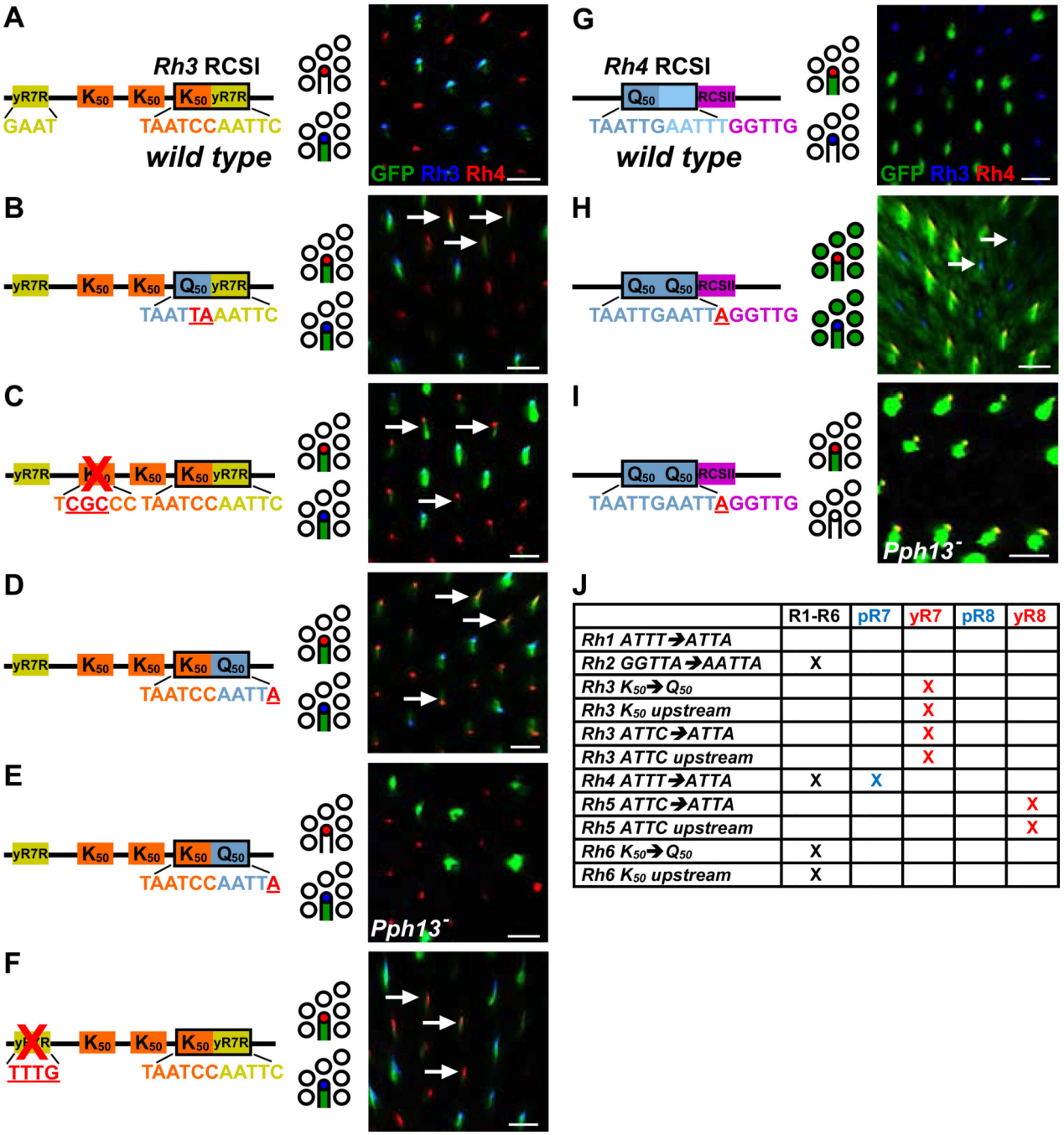
Multimerization of a P3 motif (**left**) found in broadly expressed phototransduction genes or of a specific RCSI motif (**right**) from a restricted *Rhodopsin*.

(**A, A'**) Tetramerization of a palindromic **P3** motif from *ninaC* results in broad, pan-photoreceptor expression.

(**B, B', B''**) Four copies of the ***Rh3* RCSI** that contains a Dve repressor site (orange) drive expression in R7 and R8. (**B**) The reporter is strongly biased towards **p**R7s (arrows), where *Rh3* is expressed, and faint in **y**R7s. (**B'**) GFP is expressed in all R8s, which lack Dve. (**B''**) Expression is expanded to all photoreceptors in a dve^{186} mutant background.

(**C, C', C''**) Tetramerization of the ***Rh1* RCSI** drives variable reporter expression (arrows and arrowheads in **C''**) in individual R1-R6 photoreceptors, where Rh1 is expressed (blue in **C''**). Scale bars, 10 μm.

(**D**) RCSI motifs are biased towards the respective endogenous *Rh* expression pattern (‘wild type’).



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	R1-R6	pR7	yR7	pR8	yR8
Rh1 ATTT→ATTA					
Rh2 GGTTA→AATTA	X				
Rh3 K_{50}→Q_{50}			X		
Rh3 K_{50} upstream			X		
Rh3 ATTC→ATTA			X		
Rh3 ATTC upstream			X		
Rh4 ATTT→ATTA	X	X			
Rh5 ATTC→ATTA					X
Rh5 ATTC upstream					X
Rh6 K_{50}→Q_{50}	X				
Rh6 K_{50} upstream	X				

Fig. 3. Single base pairs in the RCSI motifs of *Rh3* and *Rh4* are critical for photoreceptor subtype-specific expression

(**A**) The *Rh3* reporter is specifically expressed in **p**R7s. Left: *Rh3* promoter containing an upstream ATTC/**y**R7 repressor motif (**y**R7R), two K_{50} motifs and the *Rh3* RCSI composed of a K_{50} motif and an ATTC/**y**R7 repressor motif. (**B–F**) Mutation of the *Rh3* RCSI (**B**, **D**, **E**) or of its partial upstream repeats (**C**, **F**) causes de-repression in **y**R7s.

(**G**) The *Rh4* reporter is specifically expressed in **y**R7s. Left: *Rh4* promoter with the *Rh4* RCSI and RCSII motif (3). (**H**, **I**) Mutating a single bp in the *Rh4* RCSI causes de-repression in **p**R7 and R1-R6 that depends on *Pph13*. Scale bars, 10 μm.
(**J**) De-repression in other photoreceptor subsets (indicated by 'X') caused by mutations of RCSIs or upstream repeats.

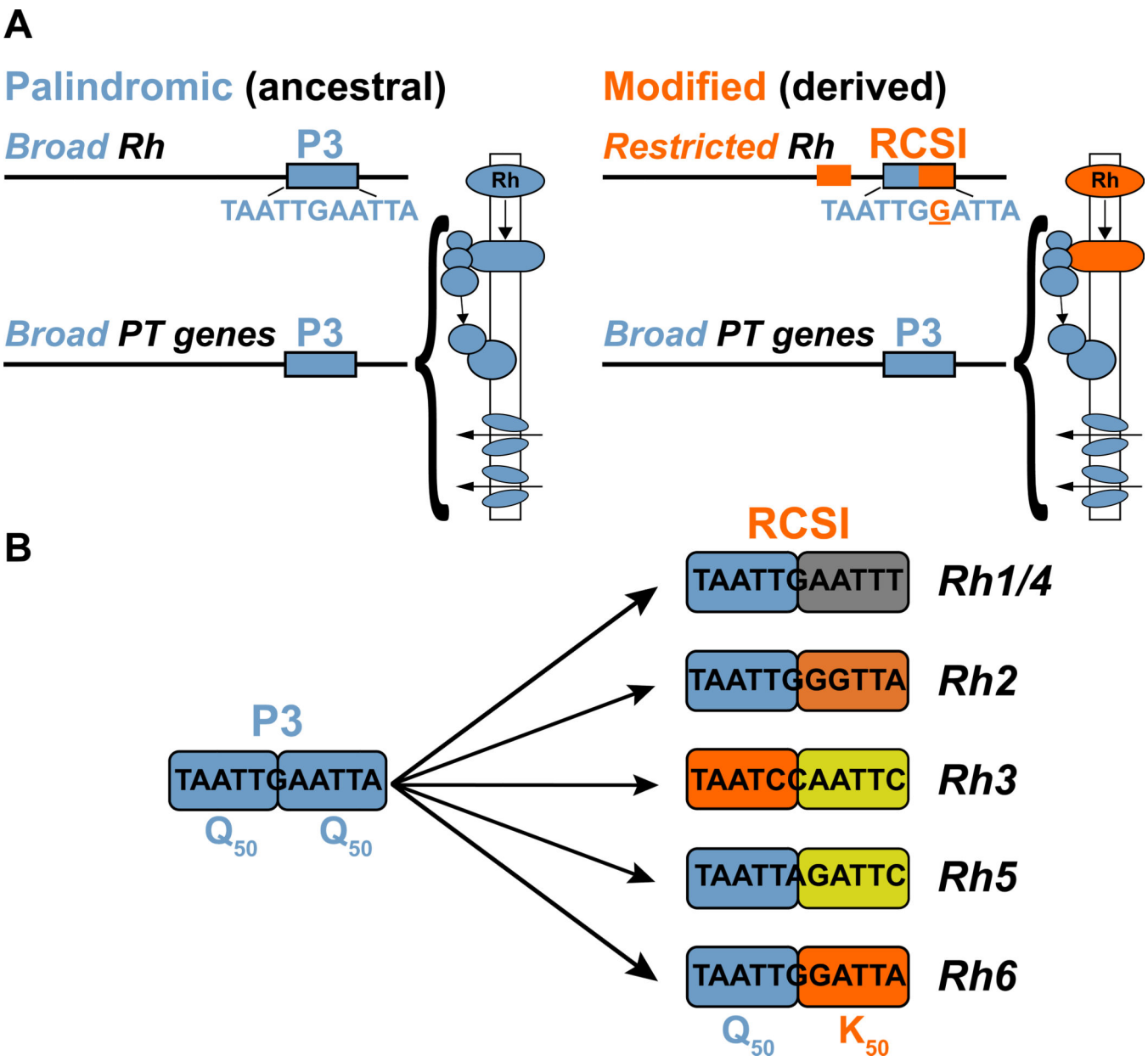

Fig. 4. Modification of a shared *cis*-regulatory motif for color vision

(**A**) A palindromic P3 motif (**TAAT**NNN**ATTA**) provides broad activation of an ancestral *Rh* and a set of phototransduction (PT) genes in all photoreceptors (left). Modification of single bps yields an RCSI motif (orange) that is essential for restricting *Rh* expression to subsets of photoreceptors (right). The upstream repetition of parts of the RCSI (orange box) is required for full subtype-specificity. Right schematic: phototransduction cascade. Note that the downstream acting factors (broad PT genes) remain expressed in all photoreceptors. (**B**) Palindromic P3 motifs bound by a photoreceptor-specific Q_{50} activator like Pph13 provide broad activation, while single bp changes in RCSI motifs specific to each *Rh* create novel activator or repressor motifs (right).