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Sequential activation of ETS proteins provides a sustained transcriptional response to EGFR signaling

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SUMMARY

How signal transduction, which is dynamic and fluctuating by nature, is converted into a stable trancriptional response, is an unanswered question in developmental biology. Two ETS-domain transcription factors encoded by the *pointed* (*pnt*) locus, PntP1 and PntP2, are universal downstream mediators of EGFR-based signaling in *Drosophila*. Full disruption of *pnt* function in developing eye imaginal discs reveals a photoreceptor recruitment phenotype, in which only the R8 photoreceptor cell type is specified within ommatidia. Specific disruption of either *pntP1* or *pntP2* resulted in the same R8-only phenotype, demonstrating that both Pnt isoforms are essential for photoreceptor recruitment. We show that the two Pnt protein forms are activated in a sequential manner within the EGFR signaling pathway: MAPK phosphorylates and activates PntP2, which in turn induces *pntP1* transcription. Once expressed, PntP1 is constitutively active and sufficient to induce target genes essential for photoreceptor development. Pulse-chase experiments indicate that PntP1 is stable for several hours in the eye disc. Sequential ETS-protein recruitment therefore allows sustained induction of target genes, beyond the transient activation of EGFR.

KEY WORDS: EGFR signaling, ETS proteins, Pointed, MAP kinase, Eye development

INTRODUCTION

Development of all multicellular organisms is dictated by a handful of signaling pathways that transmit information from the extracellular milieu and neighboring cells to the nuclei of the receiving cells. The final step in each of these pathways is the activation of transcription factors and induction of a set of target genes, the identity of which depends on the cellular context of the receiving cell. In order to make the transcriptional output sharp and resilient to noise, different pathways have adopted a common strategy that relies on a dual input. When a signaling pathway is silent, target genes are repressed by the recruitment of transcriptional repressors. Following signaling, proteins that activate transcription replace the inhibitory proteins, generating a bi-stable switch. For example, the Wnt pathway target genes bind TCF associated with the repressor Groucho/TLE, and, upon activation, Armadillo/β-Catenin replaces Groucho/TLE on TCF to trigger gene expression (Cavallo et al., 1998). Similar scenarios occur in the Hh, Notch and BMP pathways (Aza-Blanc et al., 1997; Ohlmeyer and Kalderon, 1998; Lai, 2002; Müller et al., 2003).

Following the initial burst of target-gene expression, sustained transcription requires continuous input from the signaling pathway. The inherent nature of the 'gas and brake' mechanism described above implies that, upon termination of activation, repression will follow by default. This poses a regulatory problem, as the required duration of transcription induction within the receiving cell may differ from the dynamic extracellular activation profile that triggers the signaling pathway. In such cases, it may be necessary to provide compensatory mechanisms that will propagate the initial and transient transcription-activation switch. We have examined this conundrum in the compound eye of *Drosophila*, which is patterned by repeated cycles of EGFR activation.

Development of the compound eye is a highly orchestrated process that is carried out in a sequential manner in the eye imaginal disc, to form the repeated ~750 ommatidial units, each comprised of 20 distinct cell types (Kumar, 2012). Following the posterior-to-anterior progression of a morphogenetic furrow, a single R8 photoreceptor cell is specified, independently of EGFR (Yang and Baker, 2001; Hsiung and Moses, 2002). The subsequent recruitment events include successive activation rounds of the EGFR pathway, and will give rise to all cell types of the eye (Freeman, 1996). Although the same pathway is repeatedly induced, the altered intracellular 'context' of the recruited cells will give rise to the induction of different sets of photoreceptor and accessory cell fates at each round (Banerjee and Zipursky, 1990). The limited pool of non-differentiated cells necessitates restricted activation of the EGFR pathway, which occurs in discrete bursts. Thus, several mechanisms that limit the strength of the EGFR signal have been described. These mechanisms operate at the level of active ligand production (Yogev et al., 2008), ligand diffusion (Freeman et al., 1992b; Klein et al., 2004) and, in the signal receiving cell, downstream of the receptor (Casci et al., 1999; Kramer et al., 1999; Reich et al., 1999).

The final step of the EGFR pathway, following MAP kinase (MAPK) activation, is the induction of target-gene expression, mediated by the ETS-family protein Pointed (Pnt) (Klämbt, 1993; Scholz et al., 1993; Brunner et al., 1994; Klaes et al., 1994; O'Neill et al., 1994; Gabay et al., 1996; Hsu and Schulz, 2000). Similarly, ETS proteins such as *C. elegans* LIN-1 and mammalian Elk1 are important targets of Ras/MAPK regulation (Hart et al., 2000; Yordy and Muise-Helmericks, 2000), underscoring the widespread functions of ETS proteins in Ras/MAPK signaling. *pnt* is expressed from two alternative promoters, leading to the generation of two protein isoforms, PntP1 and PntP2 (Fig. 1A) (Klämbt, 1993; Scholz et al., 1993). MAPK activity regulates activation of both Pnt isoforms, but via distinct mechanisms. The PntP2 protein is directly activated by MAPK phosphorylation, such that only the

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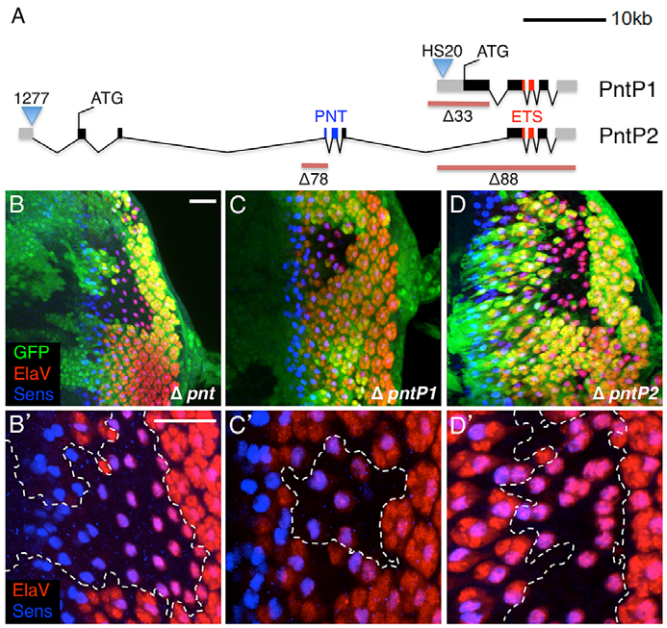

Fig. 1. Both Pnt forms are essential for photoreceptor recruitment. (**A**) The *pnt* genomic locus, which covers ~55 kb and gives rise to two different transcripts. Coding exons are marked in black and non-coding exons in gray. The *pntP1* and *pntP2* transcripts share three exons, which encode a common C-terminal region of Pnt that includes the ETS DNA-binding domain (red). The unique N-terminal region of PntP1 is encoded by a single exon, whereas five distinct exons encode the N-terminal region of PntP2, which includes the PNT domain (blue) that facilitates interaction with MAPK. The locations of the P element enhancer trap insertions HS20 and 1277 are indicated by triangles. Local excisions give rise to the $pnt^{\Delta 33}$ and $pnt^{\Delta 78}$ alleles, which specifically disrupt the *pntP1* or *pntP2* transcripts, respectively (O'Neill et al., 1994; Morimoto et al., 1996). The resulting deletions are indicated by red lines. In addition, $pnt^{\Delta 88}$ represents an excision removing parts of both transcripts (Scholz et al., 1993; Brunner et al., 1994). (**B-D′**) Homozygous *pnt* mutant clones were generated by FRT-mediated recombination using *ey-flp*. Three different *pnt* mutant alleles were used to induce the clones, visualized by the absence of a GFP signal (green). Recruited photoreceptors are marked by the general neuronal marker Elav (red), and R8 is marked by anti-Sens (blue). Loss of all photoreceptors, except for R8, was observed in all mutant clones. (B,B′) $pnt^{\Delta 88}$, a null *pnt* allele. (C,C′) $pnt^{\Delta 33}$, a specific *pntP1* mutation. (D,D′) $pnt^{\Delta 78}$, a specific *pntP2* mutation. Twofold magnifications of large clones in B-D are shown in B′-D′, respectively, with clone borders outlined. Eye discs are oriented such that anterior is towards the left. Scale bars: 10 µm.

phosphorylated form can induce transcription (Brunner et al., 1994). By contrast, PntP1 functions as a constitutively active transcription factor. However, expression of *pntP1* is restricted, and is induced by unknown transcription factors, the activity of which depends on MAPK phosphorylation (O'Neill et al., 1994; Gabay et al., 1996). In addition, the ETS repressor Yan binds to target gene enhancers, and is removed by MAPK phosphorylation (Lai and Rubin, 1992; Rebay and Rubin, 1995).

The dynamic activation profile of EGFR in the eye, and the restricted amount of ligand that is released by the photoreceptor cells, pose a challenge. Is EGFR activation sufficiently sustained and/or prominent enough to reliably induce transcriptional activation that will define the required cell fates? The current work addresses the mechanism by which consistent induction of EGFR target genes takes place, despite these limitations. We have undertaken a detailed dissection of the transcriptional responses to EGFR signaling during *Drosophila* eye imaginal disc development. Full disruption of *pnt* function revealed a complete EGFR loss-of-function photoreceptor recruitment phenotype, in which only the R8 photoreceptor cell type is specified. Ommatidia in which the function of only one of the two Pnt isoforms was eliminated showed the same R8-cell-only phenotype, thus demonstrating that each isoform is essential for photoreceptor recruitment. Further analysis showed that the two Pnt forms are activated in a sequential manner: MAPK phosphorylates and activates PntP2, which is capable only of inducing transcription of *pntP1* and not of other pathway target genes. Once expressed, PntP1 is sufficient to induce the target genes essential for photoreceptor development. The induction of EGFR-target genes may thus be sustained by PntP1 protein stability, beyond the time window where EGFR triggers local MAPK activity.

MATERIALS AND METHODS

Fly strains

The following lines were used: *GMR-Gal4*, *ey3.5-Gal4* and $tub\text{-}Gal80^{ts}$ (obtained from the Bloomington Stock Center); *pnt*-HS20 [obtained from H. Scholz (University of Cologne, Germany) and C. Klaembt (University of Münster, Germany)]; *pnt*-1277 (obtained from the Bloomington Stock Center); *rho*-X81 (Freeman et al., 1992a); *UAS-spi RNAi* (VDRC 103817); *UAS-pntP1* (obtained from C. Klaembt); *UAS-pntP2 GFP; UAS-*yan^{act} (obtained from I. Rebay, University of Chicago, IL, USA); *FRT82B arm-lacZ* (obtained from the Bloomington stock center); *ey-flp; FRT82B Ubi-GFP, FRT82B* $pnt^{\Delta 33}$, *FRT82B* $pnt^{\Delta 78}$, *FRT82B* $pnt^{\Delta 88}$ (obtained from H. McNeill, Samuel Lunenfeld Research Institute, Ontario, Canada); and ru^{PLLb} (obtained from M. Gallio, Northwestern University, Evanston, IL, USA). The information on mutations, genome organization and strains was obtained from Flybase (Tweedie et al., 2009).

DNA constructs

pntP2 GFP was generated as follows: a *pntP2* cDNA was amplified by PCR and cloned into the *Drosophila* Gateway vector pTGW (T. Murphy, Carnegie Institution of Washington), using the Gateway cloning system (Invitrogen). The resulting construct was injected into flies harboring the *AttP40* site, in order to insert the transgene into a defined genomic site.

Immunohistochemistry

Eye imaginal discs

After dissection in PBS, fixation in 4% PFA was performed. Washes were performed using 0.1% Triton X-100 (or 0.3% Triton X-100 when staining involved anti-PntP1). BSA (0.1%) was used for 15-minute blocking (or 5% NGS when staining involved anti-PntP1). An additional 30-minute blocking step with Image-it FX signal enhancer (Invitrogen Molecular Probes) was used prior to BSA blocking, when staining involved anti-β-gal or anti-PntP1. Primary antibodies were added for overnight incubation at 4°C and secondary antibodies for 2 hours at room temperature. Embryos were stained with antibodies or X-Gal according to standard procedures.

Primary antibodies

Primary antibodies used were anti-GFP (chick 1:2000; Abcam), anti-β-gal (rabbit 1:5000 after pre-absorption; Cappel), anti-Senseless (guinea pig 1:2000, obtained from H. Bellen, Baylor College of Medicine, Houston, TX, USA), anti-Eve (rabbit, 1:1000, obtained from M. Frasch, University of Erlangen-Nuremberg, Erlangen, Germany) and anti-PntP1 (rabbit 1:500, obtained from J. Skeath, Washington University, St Louis, MO, USA). Anti-Elav (rat 1:2000) and anti-Prospero (mouse 1:100) were obtained from the Developmental Studies Hybridoma Bank, University of Iowa.

Secondary antibodies

Secondary antibodies used were anti-chick DyLight 488 (1:800), anti-rat Cy3 (1:400) (obtained from Jackson ImmunoResearch), anti-rabbit Alexa405 and Alexa488, anti-mouse Alexa405 and Alexa488, and anti-guinea pig Alexa647 (1:800, obtained from Molecular Probes).

In situ hybridization

For each of the *pnt* forms, a digoxigenin (DIG)-DNA-labeled probe was prepared using a PCR-DIG probe synthesis kit (Roche). The following primers were used for amplifying probes from *pnt* cDNA.

For *pntP1*: 5′AATTCGCGCGGTGTGTG, 5′TGGACAGCGATAAGGAGCAG; 3′GGGCGCCATATCATTGAAGT5′.

For *pntP2*: 5′CGCGCGCAATATACAGCAAA; 3′AGGCGCACAATCTTGATGGA5′.

Fixation, hybridization and detection were carried out according to Kosman et al. (Kosman et al., 2004).

Simulation of transcriptional responses to EGFR activation by sequential induction of PntP2 and PntP1

The simulation is presented graphically in Fig. 6A.

The following set of ordinary differential equations were used to generate the figure.

$$\frac{\partial[Spi]}{\partial t} = \alpha_{Spi}(t) - \beta_{Spi}[Spi], \quad (1)$$

$$\frac{\partial[P2]}{\partial t} = -k(Spi)[P2] + k_{phos}[pP2], \quad (2)$$

$$\frac{\partial[pP2]}{\partial t} = k(Spi)[P2] - k_{phos}[pP2], \quad (3)$$

$$\frac{\partial[P1]}{\partial t} = \alpha(pP2) - \beta_{P1}[P1], \quad (4)$$

$$\alpha_{Spi}(t) = \begin{cases} \alpha_{Spi} & t < t_0 \\ 0 & t \geq t_0 \end{cases}, \quad (5)$$

$$k(Spi) = k_{kin}[Spi(t - \tau_p)], \quad (6)$$

$$\alpha(pP2) = \alpha_{P1}\,[pP2(t - \tau_t)]. \quad (7)$$

Boundary conditions are given by the following: $[Spi]_{t=0} = 0$; $[P2]_{t=0} = P2_{tot}$; $[pP2]_{t=0}$; $[P1]_{t=0} = 0$.

[*Spi*] is the concentration of the EGFR ligand Spitz (Spi). Spi is transiently produced at a rate α_{spi} for a time $0 < t < t_0$. Spi is then being degraded at some rate: β_{Spi}.

[*P2*] is the concentration of PntP2 protein. PntP2 is phosphorylated at a rate *k*, which depends linearly on the levels of Spi, with a time lag τ_p. This lag accounts for the time it takes Spi signal to propagate until phosphorylation produces phosphorylated PntP2 [*pP2*]. [*pP2*] is dephosphorylated at a constant rate: k_{phos}. Initially, all PntP2 is dephosphorylated and its levels are $P2_{tot}$. We assume total PntP2 levels are constant, such that $[P2]+[pP2]=P2_{tot}$ at all times.

[*P1*] is the concentration of PntP1. It is produced at a rate α, which is linearly dependent on the levels of phosphorylated PntP2, τ_t seconds earlier. τ_t represents the time it takes to produce PntP1 protein following activation of the *pntP1* gene by [*pP2*]. PntP1 is turned over at a rate β_{P1}. Parameter values are provided in supplementary material Table S1.

RESULTS

Both forms of Pnt are essential for photoreceptor recruitment

To begin our analysis of the manner by which ETS-domain transcription factors mediate EGFR signaling during *Drosophila* eye development, we sought to verify the functional requirements for Pnt in this setting. The *pnt* genomic locus gives rise to two partially overlapping transcripts, *pntP1* and *pntP2*, which are generated by distinct transcriptional promoters, positioned over 50 kb apart. The two encoded protein isoforms, PntP1 and PntP2, share a common C-terminal region, that includes their ETS DNA-binding domain. (Klämbt, 1993; Scholz et al., 1993) (Fig. 1A). We used FLP/FRT recombination to generate eye-disc clones homozygous for mutant *pnt* alleles, which affect either or both Pnt forms (Fig. 1B-D′). The alleles used represent deletions that are predicted to be molecular nulls. Full disruption of Pnt function was achieved using the $pnt^{\Delta 88}$ allele, an intragenic deletion removing the 3′ end of the *pnt* gene (Brunner et al., 1994) (Fig. 1A). As was previously shown, only R8 photoreceptors, which express both the R8-specific factor Senseless and the pan-neuronal marker Elav are specified in the complete absence of Pnt activity (Rogers et al., 2005), implying an essential requirement in recruitment of additional cells to ommatidial units (Fig. 1B,B′).

We next examined the consequences of specifically removing each of the *pnt* forms alone. To achieve this, we used the $pnt^{\Delta 33}$ and $pnt^{\Delta 78}$ alleles, small intragenic deletions that specifically disrupt the *pntP1* or *pntP2* transcripts, respectively (O'Neill et al., 1994; Morimoto et al., 1996) (Fig. 1A; supplementary material Fig. S1C-K). The form-specific clones displayed an identical phenotype to the complete removal of *pnt*, i.e. absence of photoreceptor neurons, except for R8 (Fig. 1C-D′). This result, which confirms previous observations (Yang and Baker, 2003), indicates that there is no redundancy between the two forms of Pnt, such that both are required for the establishment of EGFR-induced photoreceptor cell fates.

pntP1 but not *pntP2* transcription is dependent on EGFR signaling

Having verified an essential requirement for both forms of Pnt in mediating EGFR-based recruitment of photoreceptors, we examined the manner by which EGFR signaling influences *pnt* expression. The expression pattern of the two *pnt* forms during eye development was analyzed by following two enhancer trap lines, HS20 and 1277, integrated in the vicinity of the P1 or P2 promoters, respectively (Scholz et al., 1993) (Fig. 1A). Expression of the β-galactosidase (β-gal) reporter from these elements during embryogenesis indeed recapitulates the known distribution of the specific transcripts (Klämbt, 1993; Scholz et al., 1993) (supplementary material Fig. S1A,B).

In the eye disc, the two reporters show distinct patterns of expression. *pntP1* is expressed in a restricted manner, as revealed by HS20 (Fig. 2A,B). Expression is observed only within the differentiated region of the disc, including and posterior to the morphogenetic furrow. HS20 colocalizes with Elav in all mature ommatidia, indicating that *pntP1* expression is further confined to photoreceptor neurons. However, HS20 is also detected in cells immediately posterior to the morphogenetic furrow, only some of which express Elav. These observations imply a temporal expression pattern, consistent with the possibility that PntP1 induction leads to photoreceptor differentiation and the eventual expression of Elav (Fig. 2B). *pntP2* expression, monitored by the 1277 reporter, is expressed in a broader pattern that is not restricted to the Elav-expressing cells (Fig. 2C,D), the only exception being R8 cells, which show lower expression (Fig. 2D).

To explore the basis for the different expression patterns of the two *pnt* forms, we examined their dependence on EGFR signaling. An RNAi construct targeting the EGFR ligand Spitz (Spi) was induced, using the *ey3.5-Gal4* driver that is expressed in all eye disc cells, on both sides of the morphogenetic furrow (Fig. 2E-F′). This led to an effective block of EGFR signaling, as evidenced by the absence of Elav-positive photoreceptor cells, except for R8 cells, the specification of which does not depend on EGFR signaling (Yang and

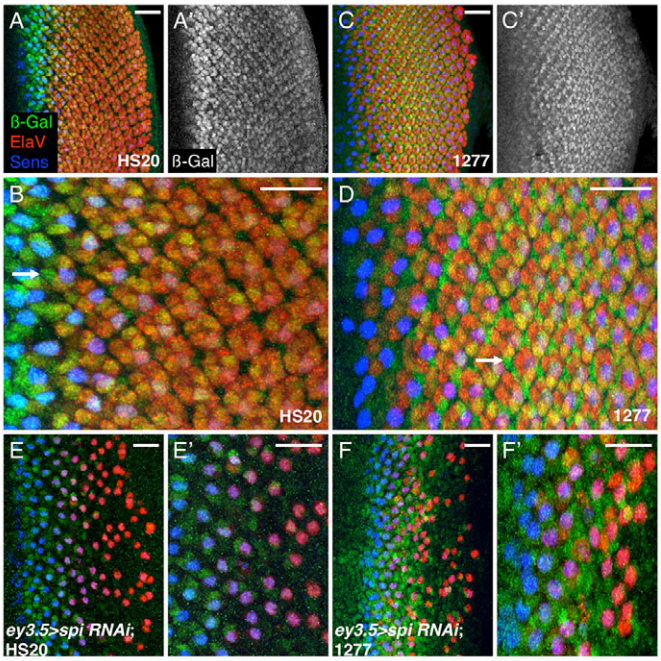

Fig. 2. *pntP1* but not *pntP2* transcription is dependent on EGFR signaling. (A-D) Expression pattern of the two *pnt* transcripts in 3rd instar larva eye imaginal discs, as revealed using the β-gal expressing enhancer trap lines HS20 (for *pntP1*) and 1277 (for *pntP2*). Panels show the β-gal reporters (green and gray), Elav (photoreceptors, red) and Sens (R8 photoreceptor, blue). (**A**,**A′**) The restricted expression pattern of HS20 in wild-type discs. (**B**) A threefold magnification of A shows a mixed expression pattern just posterior to the furrow, where some cells express only HS20 (as indicated by an arrow) and some both Elav and HS20. (**C-D**) The reporter 1277 is broadly expressed in wild-type discs, in a pattern that is not restricted to the Elav-expressing cells, as indicated by an arrow in the magnified panel (D). (**E-F′**) *pnt* reporter expression patterns in discs in which *UAS-spi RNAi* is driven by *ey3.5-Gal4*. Markers as in previous panels. Elav expression is absent from all cells except R8, owing to disruption of EGFR signaling. (E,E′) HS20 was found only in Elav-expressing cells. (F,F′) Despite complete abolishment of photoreceptor recruitment, 1277 displayed a normal expression pattern in eight rows posterior to the furrow. Eye discs are oriented such that anterior is towards the left. Scale bars: 10 μm.

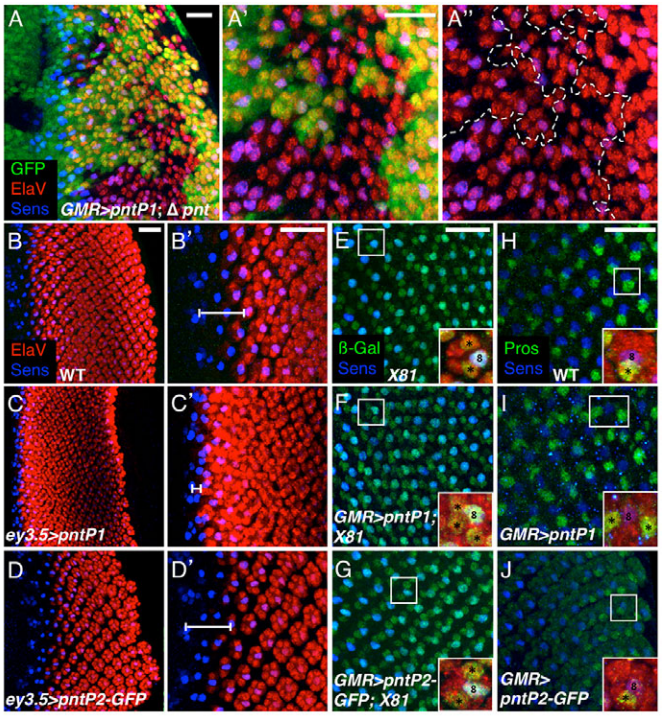

Fig. 3. PntP1 is capable of inducing ectopic photoreceptor differentiation, but PntP2 is not. (**A-A″**) Ectopic expression of *UAS-pntP1*, driven by *GMR-Gal4*, in an eye disc bearing clones of the null *pnt* mutation $pnt^{\Delta 88}$. Clones are visualized by the absence of a GFP signal (green). Photoreceptors are visualized with Elav (red) and R8 photoreceptors with Sens (blue). Multiple Elav-expressing cells surround each R8 cell. (A′,A″) Twofold magnification of A. The broken line (A″) marks the borders of the $pnt^{\Delta 88}$ clone. (**B-D′**) Ectopic expression of *UAS-pntP1* and *UAS-pntP2-GFP*, driven by *ey3.5-Gal4*, in wild-type eye discs. Photoreceptors are visualized with Elav (red) and R8 photoreceptors with Sens (blue). Scale bars in B′,C′,D′ indicate the distance between the morphogenetic furrow (MF) and the first cell clusters undergoing photoreceptor differentiation. (B,B′) Normal arrangement of developing ommatidia. (C,C′) Ectopic expression of PntP1 resulted in a disorganized pattern of Elav-expressing cells, and induction of Elav expression close to the MF. (D,D′) Ectopic expression of PntP2-GFP did not lead to any abnormalities in photoreceptor differentiation. (**E-J**) Imaginal eye disc expression patterns of X81, a β-gal expressing enhancer trap in *rho-1* (green, E-G) or of Pros (green, H-J) in wild-type discs (E,H) or in discs ectopically expressing *UAS-pntP1* (F,I) or *UAS-pntP2-GFP* (G,J), driven by *GMR-Gal4*. R8 photoreceptors are visualized in all panels using anti-Sens (blue). Insets, which include the Elav expression pattern (red), are magnifications of boxed single ommatidia, in which *rho-1*- or Pros-expressing cells are indicated by asterisks, in addition to R8. Eye discs are oriented such that anterior is towards the left. Scale bars: 10 μm.

Baker, 2001; Hsiung and Moses, 2002). This phenotype is identical to the one observed in clones deficient for *spi* (Tio and Moses, 1997).

As was observed for Elav, *pntP1* expression was retained only in R8 cells following knockdown of *spi* (Fig. 2E,E′). This indicates that, like Elav, expression of *pntP1* depends on EGFR signaling. *pntP2* expression, on the other hand, was retained in cells surrounding the R8 cells (Fig. 2F,F′). Expression was somewhat reduced in the most posterior cell rows, which we ascribe to secondary consequences of cell mis-specification and death. Therefore, *pntP2* transcription appears to be EGFR independent, consistent with its broad distribution beyond the cells where EGFR signaling normally takes place.

Taken together, these observations of the differential expression patterns of the two Pnt forms are suggestive of their modes of regulation and activity. *pntP2* is broadly expressed, independently of EGFR signaling, providing all cells with a competence to respond to MAPK activation in the restricted domains where triggering will take place. Expression of *pntP1*, however, is limited and overlapping with the domains of EGFR activation, consistent with a role for EGFR-induced MAPK activity in controlling its expression.

PntP1 is sufficient to trigger photoreceptor cell recruitment and differentiation

Having shown that both Pnt isoforms are required for photoreceptor specification, we next asked whether they are sufficient to induce EGFR target-gene expression on their own. To assess sufficiency of PntP1, we first used *GMR-Gal4*, which is broadly active posterior to the morphogenetic furrow, to drive expression of *pntP1* in eyes bearing clones of the null $pnt^{\Delta 88}$ allele (Fig. 3A-A″). Such ectopic expression of PntP1 rescued the mutant phenotype of these clones, leading to the recruitment of multiple Elav-expressing cells around the R8 photoreceptors. The typical

arrangement of photoreceptor cells in each ommatidia was lost, however, as the expression of PntP1 did not follow the orderly activation of EGFR (Fig. 3A-A″). This result demonstrates that once expressed, PntP1 can elicit photoreceptor cell fates in the absence of PntP2.

The sufficiency of PntP1 could be further demonstrated following overexpression in wild-type eye discs. As normal expression of PntP1 in the eye disc is restricted to the cells eventually expressing Elav, we used the *ey3.5-Gal4* driver to express PntP1 throughout the eye disc epithelium. Under these circumstances, the Elav expression pattern lost its highly ordered pattern, and became broad and disorganized (Fig. 3B-C′), suggesting the recruitment of additional cells. Furthermore, a global alteration in Elav expression was observed, such that Elav-positive cells are detected closer to the morphogenetic furrow, indicating premature induction (Fig. 3B′,C′).

To monitor the excessive recruitment of cells more precisely, we used markers that are specific for distinct photoreceptor types. Rhomboid 1 (Rho1) is expressed in three photoreceptors, R8, R2 and R5 (Freeman et al., 1992a), whereas Prospero (Pros) is normally expressed only in the R7 cell (Kauffmann et al., 1996). Both of these fate markers normally display a highly ordered and stereotypic array (Fig. 3E,H). Following ectopic PntP1 expression, the regular arrangement broke down, and extra cells expressing Rho1 or Pros were detected in many ommatidia (Fig. 3F,I). On average, the number of Rho1- and Pros-expressing cells rose to 3.6 cells (n=460 ommatidia) and 1.2 cells (n=317), respectively.

Similar overexpression of PntP2-GFP did not result in detectable abnormalities in photoreceptor differentiation, despite the high levels of ectopic expression (Fig. 3D,G,J; supplementary material Fig. S2). We argue that as the activity of PntP2 depends on MAPK phosphorylation, ectopic expression in cells that are not immediately adjacent to the normal source of Spi, will have no consequences. Taken together, these experiments support a scenario of sequential activation of Pnt forms, as part of the EGFR signaling pathway. In this scheme, the broadly expressed PntP2 is first locally activated by MAPK, and subsequently triggers (directly or indirectly) the expression of PntP1, which then acts constitutively to induce pathway targets in the signal-receiving cells.

Expression of *pntP1* requires induction by PntP2

We next examined the role of PntP2 in the induction of *pntP1* expression. The HS20 reporter for *pntP1* expression is inserted in the locus, and thus could not be used in *pntP2* mutant clones. We therefore followed expression of PntP1 protein, using an antibody that is specific to this form (Alvarez et al., 2003). PntP1 protein is most prominent in the proneural clusters ahead of the morphogenetic furrow, and shows a weaker expression posterior to the furrow. This pattern is similar to the distribution of activated MAPK, as monitored by dpERK antibodies (Gabay et al., 1997), and is therefore consistent with the induction of *pntP1* expression by EGFR/MAPK signaling. As expected, PntP1 protein is not detected by the form-specific antibody in *pntP1* mutant clones (Fig. 4A-A″). We then generated mutant clones for *pntP2*, and followed the expression of PntP1 protein in a similar manner. Both the prominent expression of PntP1 in the proneural clusters, as well as its expression posterior to the furrow were eliminated in these clones, demonstrating that PntP2 is required for induction of *pntP1* expression (Fig. 4B-B″)

In view of the requirement for transcriptional activation of *pntP1* by the PntP2 ETS protein, we asked whether PntP1 can also maintain its own expression. Ectopic expression of PntP1 leads to excessive photoreceptor differentiation (Fig. 4C,C″), but this is not accompanied by elevation in the level of the *pntP1* transcriptional reporter HS20 (Fig. 4C″). Importantly, this result suggests that once PntP1 is expressed, the activity it provides is not self-sustaining. Rather, it depends on the stability of *pntP1* RNA and protein that was produced initially, following the burst of activated MAPK and PntP2 activation. Similarly, ectopic expression of PntP1 did not elevate the expression levels of the *pntP2* transcriptional-reporter 1277 (Fig. 4D-D″).

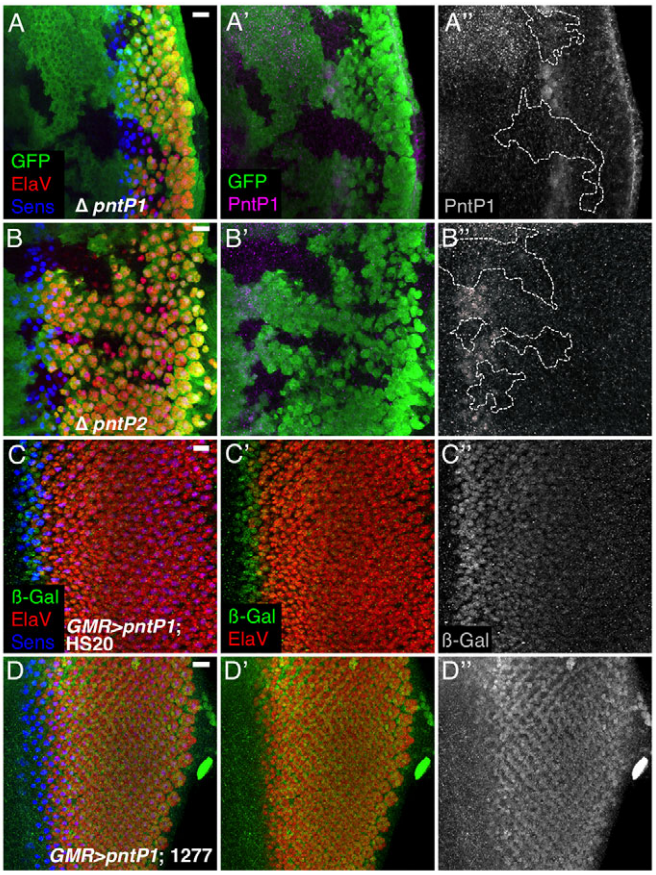

Fig. 4. Expression of PntP1 requires induction by PntP2. (**A-B″**) PntP1 expression in the absence of *pntP1* or *pntP2*. PntP1 protein was visualized using anti-PntP1 antibodies in eye discs bearing *pntP2*-specific mutant clones. (A,B) Clones are marked by the absence of a GFP signal (green). Photoreceptors were visualized with Elav (red) and R8 photoreceptors with Sens (blue). Absence of PntP1 (magenta or gray) is detected in clones (marked by dashed line) where *pntP1* (A″) or *pntP2* (B″) were deleted. (**C-D″**) Ectopic expression of *UAS-pntP1* driven by *GMR-Gal4*. The form-specific transcriptional reporters HS20 for *pntP1* (C) or 1277 for *pntP2* (D) were visualized to monitor expression pattern and levels of the endogenous transcripts (green, gray). Photoreceptors were visualized with Elav (red) and R8 photoreceptors with Sens (blue). In both cases, no significant alteration in endogenous expression levels was detected. Eye discs are oriented such that anterior is towards the left. Scale bars: 10 μm.

Sequential activation of Pnt proteins compensates for low EGFR activation levels

A sequential scenario, in which PntP1 expression is dependent on PntP2 activation, can readily explain the *pnt*-null phenotype observed when only the PntP2 form is inactivated. Less clear, however, is the insufficiency of PntP2, which appears to be

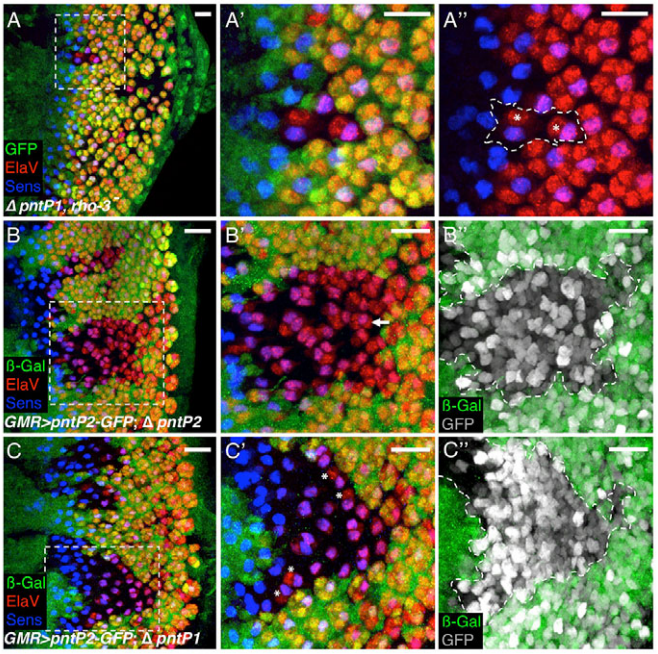

Fig. 5. EGFR hyperactivation or high levels of PntP2 can substitute for loss of PntP1. (**A-A″**) *pntP1*-specific mutant clones, visualized by the absence of a GFP signal (green), were induced in the background of *rhomboid-3* mutant flies. (A′,A″) Enlargements of the boxed clone in A. Photoreceptors are visualized with Elav (red) and R8 photoreceptors with Sens (blue). Asterisks in A″ mark instances of photoreceptor recruitment rescue events, which were associated with 6.0% (*n*=232) of mutant ommatidia. (**B-C″**) *UAS-pntP2-GFP* was ectopically expressed via the *GMR-Gal4* driver, in eye discs bearing mutant clones of either *pntP2* (B-B″) or *pntP1* (C-C″). Clones are visualized by the absence of a β-Gal signal (green). Photoreceptors are visualized with Elav (red) and R8 photoreceptors with Sens (blue). B′,B″ and C′,C″ are enlargements of the boxed clones in B and C, respectively. The expression pattern of PntP2-GFP is shown in gray in B″ and C″. Arrow in B′ marks prominent photoreceptor recruitment rescue. Asterisks in C′ indicate instances of photoreceptor recruitment in the *pntP1* mutant background, which were associated with 15.7% (*n*=226) of mutant ommatidia. Eye discs are oriented such that anterior is towards the left. Scale bars: 10 μm.

incapable of inducing photoreceptor cell recruitment and differentiation on its own. This insufficiency may arise from distinct transcriptional activation capacities of the two Pnt forms, which may associate with different enhancer-binding proteins. An alternative possibility is that levels of MAPK-phosphorylated PntP2 within signal-receiving cells can support only *pntP1* expression, but not other pathway targets.

Expression of PntP1 or PntP2 alone is commonly sufficient to trigger expression of EGFR and FGFR target genes (Gabay et al., 1996; Samakovlis et al., 1996; Alvarez et al., 2003; Zhu et al., 2011). The unusual reliance on sequential activation of both Pnt forms during eye disc development may reflect the low and transient levels of EGFR activation that are induced at each round of photoreceptor recruitment. If this were the case, endogenous PntP2 may suffice for target-gene induction under circumstances of elevated levels of EGFR signaling in the eye disc.

To address this issue, we generated and examined mutant *pntP1* clones in eye discs also mutant for *rho-3*. EGFR signaling is elevated in this background, owing to more effective production of the active form of the EGFR ligand Spi (Yogev et al., 2008). Indeed, in these double-mutant clones, recruitment of Elav-expressing cells was observed in some of the ommatidia (Fig. 5A-A″). This observation indicates that PntP2 is mechanistically capable of inducing photoreceptor cell fates on its own. In addition, it supports the notion that the requirement for sequential recruitment of Pnt proteins stems from the low and transient nature of EGFR activation in the eye disc.

Hyperactivation of PntP2 can substitute for loss of PntP1

If PntP2 can be mechanistically sufficient for induction of target genes, raising the levels of PntP2 may also allow it to induce target genes in the absence of PntP1. To examine this possibility, the *GMR-Gal4* driver was used to overexpress PntP2-GFP. We first determined that this construct was functional, by demonstrating restoration of Elav expression in mutant clones for *pntP2* (Fig. 5B-B″). The same construct was then expressed in the background of $pnt^{\Delta 33}$ mutant clones, in which *pntP1* is specifically impaired (Fig. 5C-C″).

Partial rescue was observed in the *pntP1*-mutant background, in that numerous ommatidia displayed recruitment of one or two cells expressing Elav, in addition to R8 (Fig. 5C′). High levels of PntP2 can therefore elicit photoreceptor recruitment and differentiation on their own, in cells where MAPK activation takes place. It is important to note that this experiment is conceptually distinct from ectopic PntP2 expression in a wild-type background, where no ectopic photoreceptor induction was observed (Fig. 3D,G,J). Here, we demonstrate that higher levels of PntP2 in cells that are normally exposed to the endogenous MAPK activation, can substitute for the absence of PntP1 and give rise to Elav expression.

Simulation of sequential recruitment of Pnt proteins

Sequential activation of PntP2 and PntP1 implies that the duration of transcriptional activation shifts from a regimen that relies on PntP2 protein phosphorylation and dephosphorylation, to one that is dependent only on PntP1 RNA and protein stability. To examine the effect of such a shift on the time frame of transcriptional induction of target genes, the sequential induction of Pnt proteins was described by differential equations (Materials and methods), and parameters were chosen based on an estimate of biologically relevant values (supplementary material Table S1).

Signal transduction and phosphorylation are more rapid events than protein production and turnover rates. The simulation shows that kinetics of EGFR activation by Spi, and persistence of the phosphorylated active form of PntP2, are transient (less than 20 minutes). However, the induction of PntP1 expression sustains the biological response for 2-3 hours, as long as PntP1 RNA and protein are stable (Fig. 6A and supplementary material Table S1).

PntP1 protein stability

We sought to measure the stability of PntP1 protein in the context of the developing eye disc, as a means of demonstrating that the sequential signaling scenario is feasible. To achieve this, we used $Gal80^{ts}$, a temperature-sensitive variant of the Gal4 repressor Gal80 (McGuire et al., 2004). Expression of *pntP1* was induced in the eye by *GMR-Gal4* at 29°C, the restrictive temperature for $Gal80^{ts}$. Eye discs were fixed at different time intervals following a shift down to 18°C, and stained for PntP1 protein. The endogenous level of PntP1 protein posterior to the morphogenetic furrow is low, and represents only 8.3% of the total level that was observed after induction of *pntP1*. Even after a 10-hour chase,

significant levels of ectopic PntP1 could be detected. The estimated half-life of PntP1 under these conditions is about 6 hours. Stability of PntP1 protein is therefore sufficient to promote the duration of response to EGFR signaling, irrespective of the transient and fluctuating level of the signal.

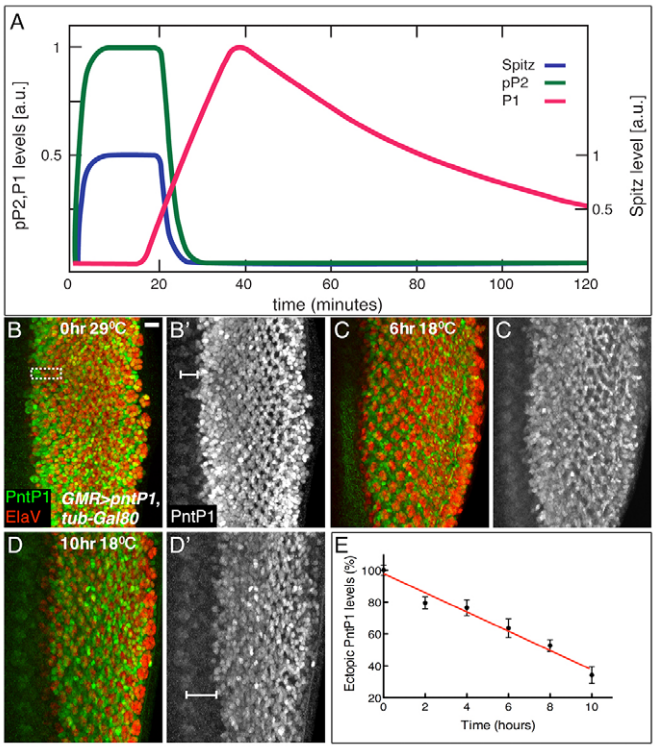

Fig. 6. Stability of PntP1 is sufficient to support effective transcription by Pnt proteins. (**A**) Simulation of the temporal profile of Pnt protein levels, based on estimated biological values, when sequential activation of the two Pnt isoforms is triggered in response to EGFR signaling. Transient EGFR activation by Spitz (Spi, blue) leads to a parallel elevation in the activation of MAPK and phosphorylation of PntP2 (pP2, green). Induction of *pntP1* expression (P1, red) by phosphorylated PntP2 prolongs the transcriptional response, in a manner that depends upon the stability of *pntP1* mRNA and protein. The parameters and equations used are presented in the Materials and methods, and in supplementary material Table S1. (**B-D′**) Ectopically expressed PntP1 protein remains stable for several hours. *UAS-pntP1* was ectopically expressed via the *GMR-Gal4* driver in developing eye imaginal discs at 29°C. Temporal control was made possible by incorporating constitutively expressed Gal80^{ts}, a temperature-sensitive repressor of Gal4 (McGuire et al., 2004). PntP1 protein (green, gray) was visualized using anti-PntP1 antibodies, at 2-hour intervals after cessation of ectopic PntP1 expression, achieved by shifting of larvae to 18°C, the permissive temperature for Gal80^{ts} activity. The PntP1 expression pattern is shown in representative images of discs dissected and stained at 0 hours (B,B′), 6 hours (C,C′) and 10 hours (D,D′) following the shift. The photoreceptor field was visualized with anti-Elav (red). Bars in B′,D′ indicate the extent of newly differentiated anterior disc regions, in which only the relatively weak levels of endogenous PntP1 are detected, which progressively widen following Gal80^{ts} activation. Scale bar: 10 μm. (**E**) Dynamics of PntP1 degradation determined by quantification of ectopic PntP1 levels. Image J software (NIH) was used to determine fluorescence intensity in 300 μm^2 rectangular areas at the anterior-most region of ectopic PntP1 expression (dashed box in B). The values plotted in the graph represent average intensities at the indicated time-points from five different eye discs, relative to the value at 0 hours. The intensity of endogenous PntP1 was subtracted in all cases. Eye discs are oriented such that anterior is towards the left.

DISCUSSION

This study demonstrates that both forms of the Pnt ETS protein are required for induction of photoreceptor cell fates, and that the requirement is sequential. Our interpretation of the Pnt-based response in EGFR signal-receiving cells of the eye imaginal disc is as follows (see also scheme in Fig. 7A-C). PntP2 activity is dependent on EGFR/MAPK activation, and possesses a low capacity to trigger target genes, owing to insufficient duration of the activated phosphorylated form. This transient activation of PntP2 is sufficient, however, to trigger (directly or indirectly) expression of *pntP1*. Signaling within the cell is then propagated, irrespective of the activation of EGFR, as long as the PntP1 protein persists. When the expression of PntP1 was driven independently of EGFR signaling, it induced photoreceptor cell fates on its own, supporting the notion that PntP1 transcriptional activity constitutes a second tier of the cellular response. Sequential activation may also provide amplification of the signal, if each molecule of PntP2 is capable of triggering several rounds of *pntP1* transcription.

Owing to the different modes of Pnt activation, the sequential use of the two forms impinges on the kinetics of signaling. Phosphorylation of PntP2 provides the first layer of response to phosphorylation by MAPK, but may be short-lived, owing to dephosphorylation and the transient nature of EGFR activation within the eye disc. It should also be noted that the activated form of MAPK may be transient, owing to the activity of MAPK phosphatases that were shown to attenuate EGFR signaling in the eye and in other tissues (Kim et al., 2004). By induction of *pntP1* expression, the response is no longer dependent on the regimen of MAPK phosphorylation, and relies solely on the stability of PntP1 mRNA and protein, thus extending its duration. This scenario ensures that the required target genes will be induced appropriately. As PntP1 does not trigger its own expression, this response is eventually terminated.

The sequential Pnt activation mechanism may be especially crucial in the eye disc, where the levels of EGFR activation are low due to the attenuation of the amount of active ligand that is secreted. Thus, low levels of Spi maintain a short range of signaling. In parallel, negative-feedback loops, including Argos and Sprouty, are

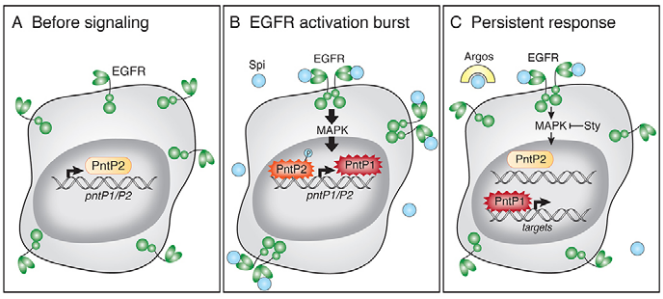

Fig. 7. Sequential activation of Pnt proteins compensates for low and transient EGFR activation levels. (**A**) Prior to the onset of signaling, photoreceptor precursors express inactive PntP2 protein. (**B**) Upon initiation of signaling, the secreted ligand Spi binds the EGF receptor on the receiving cell. The signal is relayed via MAPK phosphorylation of PntP2, which in this activated form induces *pntP1* transcription. (**C**) The constitutively active PntP1 protein accumulates and functions in the nucleus to induce target gene expression. Although EGFR signaling is attenuated by reduced production of Spi and negative-feedback responses (Argos and Sprouty), PntP1, which is stable, makes the cells refractive to signaling attenuation, and continues to induce target genes that are crucial for photoreceptor recruitment.

induced by EGFR signaling. The expression of PntP1 would make the cells that already responded to EGFR activation refractive to the negative signals they are now producing. Thus, PntP1 serves to propagate the EGFR transcriptional response, irrespective of the dynamic changes in EGFR/MAPK signaling. Although the cell is still dependent upon the perdurance of the PntP1 protein, photoreceptor cell fates will be appropriately induced, as PntP1 is sufficiently stable to trigger the crucial responses.

In various other developmental scenarios, each of the Pnt isoforms is expressed in a distinct set of tissues and functions on its own within that context. For example, PntP1 is expressed in the embryonic neuroectoderm, whereas PntP2 is expressed in the mesoderm (Klämbt, 1993; Gabay et al., 1996). It will be interesting to explore whether a sequential mechanism, similar to the one described in the eye, is operating in other contexts where the two Pnt forms are expressed within the same tissue, such as during EGFR-dependent recruitment of chordotonal cells in the leg disc (zur Lage and Jarman, 1999; zur Lage et al., 2004). As in the eye disc, EGFR activation in chordotonal precursors occurs in discrete bursts generated by a relay mechanism, in which a recruited cell becomes a source for new ligand. It will also be important to identify the transcription factor(s) that are phosphorylated by MAPK, and induce the expression of *pntP1* in tissues where PntP2 is absent. It is interesting to note that sequential activation of ETS proteins was recently reported for the process of reprograming amniotic cells to endothelial cells (Ginsberg et al., 2012). However, it is not known whether the second cohort of ETS proteins that are recruited in this sequence are constitutively active in the same way as PntP1.

Mechanistically, PntP2 is capable of inducing the set of target genes that represent acquisition of photoreceptor cell fates. When boosting the signaling activity, either by elevating EGFR signaling in a *rho-3* mutant background or upon overexpression of PntP2, this form alone is capable of partially inducing photoreceptor cell fates. We assume that, under normal conditions, activation of PntP2 is too transient to induce the necessary target genes. What makes the induction of *pntP1* transcription more responsive to activated PntP2, and how it avoids perturbation by noise and fluctuations in PntP2 activation, are interesting questions for future study.

Although the two Pnt isoforms share the same DNA-binding domain, their distinct transcription-activation domains may impinge not only on their mode of activation, but also on different preferences for association with other transcription factors and thus on the choice of genes that are induced. We believe that the composition of other transcription factors, expressed by a given cell will determine whether PntP2 will activate the expression of *pntP1*. Indeed, there are tissues such as the embryonic mesoderm, where only *pntP2* is expressed and does not induce expression of *pntP1* (Klämbt, 1993).

The sequential activation of Pnt proteins presented here provides another solution to the perdurance of gene expression. The ability of a signal-induced transcription factor to maintain its own expression gives rise to prolonged expression. Alternatively, long-term inactivation of a transcriptional repressor can provide an effective mechanism. For example, transient MAPK activation was shown to induce long-term phosphorylation that inactivates the Groucho co-repressor (Helman et al., 2011).

Transcriptional responses by the canonical developmental signaling pathways rely on a ‘gas and brake’ model, where constitutive repression of transcription of the relevant target genes is overcome by triggering the activity of key transcription factors, to induce gene expression. This implies not only a direct link between the signaling cascade and the onset of transcription, but also a continuous obligatory coupling between the two. When the extracellular signaling regimen is transient, this coupling poses a problem for sustained and reliable transcription, and subsequent induction of cell fates.

In the case of ETS proteins, activation of transcription by RTKs requires simultaneous inactivation of an ETS repressor termed Yan (Lai and Rubin, 1992; Rebay and Rubin, 1995) and activation of ETS activators, which compete for the same binding sites on the DNA. The mechanism of sequential activation of Pnt protein forms presented in this work provides one solution for sustaining the transcriptional response, and is based on the distinctly different mode of activation of each Pnt form. Another advantage is that a constitutively active transcription factor represents a form that is refractive to negative-feedback responses to signaling that it may induce, assuring a sustained transcriptional response of the activated cells. All other *Drosophila* ETS proteins are similar to PntP2: they contain the Pnt domain that mediates MAPK association as well as putative phosphorylation sites, and are likely to be activated directly by MAPK phosphorylation (Tweedie et al., 2009). Acquisition of the PntP1-specific exons may have endowed the *pnt* locus with the unique capacity of sustained induction of target genes. With this paradigm in mind, it would be interesting to explore whether transcriptional responses of other signaling pathways may use such a two-tier mechanism to propagate signaling within the receiving cells under conditions where the extracellular signal is transient.

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Competing interests statement
The authors declare no competing financial interests.

Author contributions
A.S. participated in planning and executing the experiments, and in writing, S.Y. participated in planning and executing the experiments; E.D.S. and B.-Z.S. participated in planning the experiments and in writing.

Supplementary material
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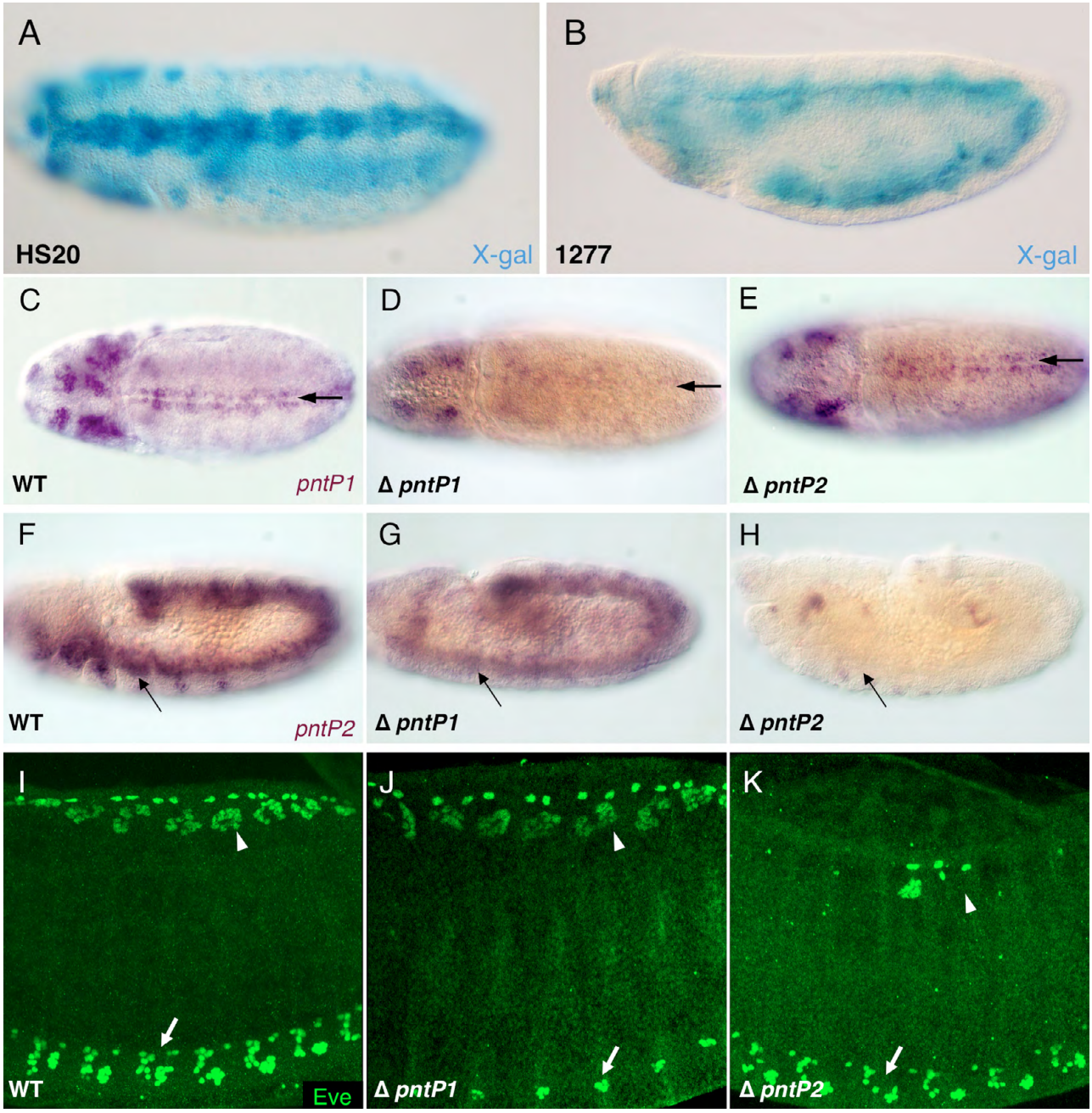

Fig. S1. HS20 and 1277 faithfully display the expression pattern of each *pnt* form, whereas $pnt^{\Delta 33}$ and $pnt^{\Delta 78}$ mutants represent specific disruptions of each of the *pnt* transcription units. (**A**,**B**) Expression pattern of the two enhancer traps in stage 10-11 embryos, monitored by X-gal staining. HS20 (*pntP1)* is detected in the ventral ectoderm (A) while 1277 (*pntP2)* is detected in the mesoderm of the embryo (B). (**C-E**) *In situ* hybridization of a *pntP1*-specific probe to stage 10-11, wild-type (C), mutant *pntP1* ($pnt^{\Delta 33}$) (D) and mutant *pntP2* ($pnt^{\Delta 78}$) (E) embryos. Arrows indicate the ventral midline. A disruption of *pntP1* expression was observed in 23% of the examined embryos (*n*=52) from a cross between $pnt^{\Delta 33}$ heterozygotes, but only in 2% of the examined embryos (*n*=86) from a cross between $pnt^{\Delta 78}$ heterozygotes. (**F-H**) *In situ* hybridization of a *pntP2*-specific probe to stage 10-11, wild-type (F), mutant *pntP1* ($pnt^{\Delta 33}$) (G) and mutant *pntP2* ($pnt^{\Delta 78}$) (H) embryos. Arrows indicate the mesoderm. Fewer than 5% of the embryos (*n*=65) from a cross between $pnt^{\Delta 33}$ heterozygotes showed abnormality in *pntP2* expression, while expression was strongly reduced or missing in 21% of the examined embryos (*n*=74), from a cross between $pnt^{\Delta 78}$ heterozygotes. (**I-K**) Anti-Eve staining of stage 14 wild-type (I), mutant *pntP1* ($pnt^{\Delta 33}$) (J) and mutant *pntP2* ($pnt^{\Delta 78}$) (K) embryos. Arrowheads indicate DA1 muscle clusters and arrows indicate the CNS. Approximately one quarter of the embryos from a cross between $pnt^{\Delta 33}$ heterozygotes (*n*=83) showed a strong reduction in Eve staining of the CNS, while the mesodermally derived DA1 clusters were unaffected (J). Conversely, DA1 cluster staining was nearly absent in approximately one quarter of the embryos from a cross between $pnt^{\Delta 78}$ heterozygotes (*n*=120). CNS staining was only weakly affected in these embryos (K).

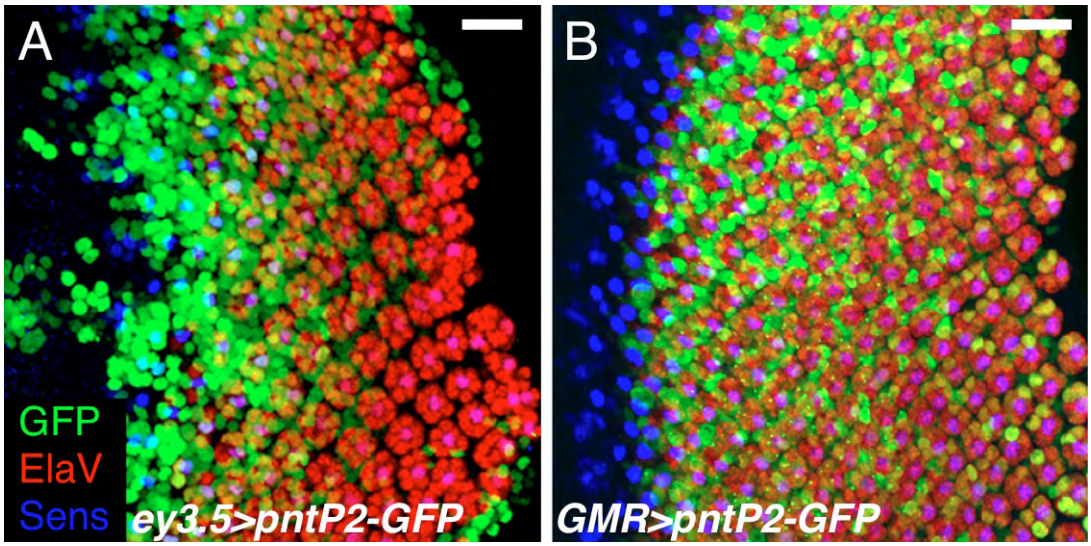

Fig. S2. The *UAS-PntP2* GFP-tagged construct is robustly expressed. (**A**,**B**) Imaginal eye discs expressing *UAS-pntP2-GFP* via the *ey3.5-Gal4* driver, which is active throughout the eye disc epithelium (A), or via *GMR-Gal4*, which is active posterior to the morphogenetic furrow (B). Photoreceptors are visualized with Elav (red) and R8 photoreceptors with Sens (blue). Visualization of PntP2-GFP using anti-GFP (green) attests to the robust levels of expression obtained using either Gal4 driver.

Table S1. Parameters used for the graph in Fig. 6A

Parameter	Physical meaning	Value
α_{Spi}	Spi production rate	10^{-3} seconds$^{-1}\mu M^{-1}$
β_{Spi}	Spi degradation rate	10^{-2} seconds^{-1}
t_0	Time when Spi values begin to drop	10^3 seconds
k_{kin}	Spi dependent PntP2 phosphorylation rate	10 seconds$^{-1}\mu M^{-1}$
τ_p	Time for the propagation of the Spi signal until PntP2 is phosphorylated	15 seconds
k_{phos}	pPntP2 dephosphorylation rate	10^{-1} seconds^{-1}
α_{P1}	pPntP2-dependent PntP1 production rate	10^{-2} seconds$^{-1}\mu M^{-1}$
τ_t	Time to produce PntP1 protein following activation by pPntP2	900 secondsa
β_{P1}	PntP1 turnover rate	1/3600 seconds^{-1}
$P2_{tot}$	Total PntP2 levels	10 μM

The time scales and lag times of phosphorylation and dephosphorylation of PntP2 are much faster than those of PntP1. This reflects the fact that signal transduction and phosphorylation are faster events than protein production and turnover rates.