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Identification of Three Required Positive Cis-Regulated Inputs of the Sea Urchin Pigment Cell Gene Polyketide Synthase 1

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IDENTIFICATION OF THREE REQUIRED POSITIVE *CIS*-REGULATORY INPUTS OF THE SEA URCHIN PIGMENT CELL GENE POLYKETIDE SYNTHASE 1

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ABSTRACT

Sea urchin pigment cells are single cells of mesodermal origin embedded in the aboral ectoderm. *Strongylocentrotus purpuratus* polyketide synthase 1 (Sp-PKS1) is required for the biosynthesis of the echinochrome pigment. Evidence suggests that pigment cells are immune cells. In order to reconstruct the gene regulatory network of pigment cells a bottom-up approach combined with comparative genomics has been used in this study. We compared the *cis*-regulatory regions of five pigment cell genes, *Sp-Pks1*, *flavin monooxygenase 1*, *2*, and *3* (*Sp-Fmo*) and *sulfotransferase* (*Sp-Sult*), across three different species, *Strongylocentrotus purpuratus*, *Mesocentrotus franciscanus*, and *Strongylocentrotus fragilis*. The computational tool used was multiple expectation maximization motif elicitation analysis. Thirty *cis*-regulatory motif candidates were identified, three of which were considered for further analysis. The functionality of these motifs was tested by injecting embryos with a *-2KbPks-Gfp* DNA construct having one of the three motifs mutagenized. All three motifs resulted to be functional *cis*-regulatory sequences. Specifically, they contained DNA-binding sites for transcriptional activators of *Sp-Pks1*.

Keywords: polyketide synthase, sea urchin, pigment cells, transcriptional *cis*-regulation, differentiation, gene regulatory networks

INTRODUCTION

The broad objectives of this study were to gain further knowledge on the gene regulatory networks (GRNs) that regulate cell-type specification and differentiation during embryo development. The development of sea urchin embryo pigment cells was used as the research model. To uncover the structure of the GRN regulating the

specification and differentiation processes of pigment cells a bottom-up approach was used, starting with the analysis of the *cis*-regulatory sequence of a differentiation gene (Calestani and Rogers 2010). Specifically, through the integration of computational and experimental approaches, we aimed to characterize the *cis*-regulatory architecture of *Sp-Pks1*, a gene required for the biosynthesis of the larval pigment echinochrome (Calestani et al. 2003). This work will help to uncover the gene regulatory connections between cell specification and the terminal process of cell differentiation, which are poorly understood.

The pigment cells of the sea urchin larvae are of mesodermal origin, specifically they develop from the non-skeletogenic mesoderm cells (NSM; Cameron et al. 1991; Ruffins and Ettensohn 1996). Pigment cells are the first out of the four NSM types to be specified. Pigment cell precursors migrate into the blastocoel during the early gastrula stage and embed in the ectoderm by the pluteus stage (Gustafson and Wolpert 1967; Gibson and Burke 1985; Kominami et al. 2001). The specification process of pigment cells is triggered by a Delta/Notch (D/N) signaling pathway (Sherwood and McClay 1999; Sweet et al. 1999, 2002; McClay et al. 2000; Oliveri et al. 2002; Croce and McClay 2010; Materna and Davidson 2012). Another factor required for the specification of pigment cells and other NSM cells is the membrane protein Numb, which acts synergistically with Notch (Range et al. 2008). At the 7th cleavage stage the D/N signaling directly activates the transcription factor *gcm*, which is expressed in a ring of Veg2 cells and is essential for pigment cell development (Ransick et al. 2002; Ransick and Davidson 2006). By the end of the mesenchyme blastula stage, pigment cell precursors are restricted to the aboral NSM by a regulatory process involving Nodal signaling from the oral ectoderm and repression of aboral NSM genes by oral NSM genes (Duboc et al. 2010; Materna et al. 2013). Vice versa, there is also a repression of oral NSM genes by aboral NSM genes (Solek et al. 2013; Materna et al. 2013). Studies have shown that other cell signaling pathways are involved in the NSM development; Hedgehog signaling from the endoderm is required to develop the normal number of pigment cells (Walton et al. 2009); later in development Eph/Ephrin signaling from the aboral and ciliary band ectoderm is required for the migration of pigment cells, insertion into the ectoderm, and acquisition of the stellar shape typical of pigment cells (Krupke et al. 2016). Moreover, a transcription factor belonging to the E-proteins family, SpE-Alt, is required for the correct timing of pigment cell precursor ingression into the blastocoel (Schrankel et al. 2016). *SpE-Alt* does not seem to be a regulator of *Sp-Pks1* since *Sp-Pks1* is still expressed in *SpE-Alt* knock-down embryos (Schrankel et al. 2016). The regulatory relationships of *SpE-Alt* with the cell signaling described above have not been characterized.

Experimental evidence suggests that pigment cells have a role in the immune system of sea urchin larvae (Service and Wardlaw 1984; Gibson and Burke 1987; Hibino et al. 2006; Castoe et al. 2007; Kiselev et al. 2013; Solek et al. 2013; Ho et al. 2016; Schrankel et al. 2016; Buckley et al. 2017). Pigment cells synthesize the echinochrome A pigment, which has been shown to have antimicrobial properties and produce hydrogen peroxide when oxidized (Perry and Epel 1981; Service and Wardlaw 1984; Brasseur et al. 2017). Pigment cells migrate to the gut when larvae are in seawater containing the bacterium *Vibrio diazothrophicus* (Ho et al. 2016). This immune response is mediated by members of the IL17 cytokine family (Buckley et al. 2017). Moreover, pigment cells interact with other immune cells of the larva such as globular, amoeboid, and filopodial

cells, especially in the proximity of bacteria and in response to wounding (Ho et al. 2016). *Sp-Pks1* is required for echinochrome A biosynthesis and is exclusively expressed in pigment cells and their precursors starting between 15 and 18 h in *S. purpuratus* (Calestani et al. 2003). At the blastula stage, *Sp-Pks1* is expressed in a ring of about 20 NSM precursors surrounding the skeletogenic mesoderm (SM) and by the end of the mesenchyme blastula stage is restricted to the aboral NSM (Calestani et al. 2003). At the gastrula stage, *Sp-Pks1* expression is detected in cells migrating into the blastocoel (Calestani et al. 2003). Gene expression is maintained throughout the pluteus stage in cells embedded in the aboral ectoderm, coincident with the distribution of pigment cells (Calestani et al. 2003; Gibson and Burke 1985; Cameron et al. 1991; Ruffins and Ettensohn 1996).

With an integration of classical promoter deletions and comparative genomics approaches a previous study led to the identification of 500 bp (between -1.5 and -1Kb) that are required for the correct spatial and temporal expression of *Sp-Pks1* (Calestani and Rogers 2010). Within this *cis*-regulatory region, our study determined that the transcription factors GATAE, GCM, and KRL-LIKE, known to be expressed in pigment cell precursors, are direct positive regulators of *Sp-Pks1* (Calestani and Rogers 2010).

In this work, in order to predict DNA-binding sites for additional regulators of *Sp-Pks1,* we performed a comparative genomics analysis by using multiple expectation maximization motif elicitation (MEME; Bailey and Elkan 1994). Putative *cis*-regulatory sequences from three sea urchin species, *Strongylocentrotus purpuratus* (SP), *Mesocentrotus franciscanus* (MF), and *Strongylocentrotus fragilis* (SF) were used. To increase the predictive power of our comparative genomics analysis we included other pigment cell differentiation genes coregulated by GCM and GATAE, hence they are likely to belong to the same differentiation gene battery, *flavin monooxygenase* (*Sp-Fmo*) *1*, *2*, and *3*, and *sulfotransferase* (*Sp-Sult*; Calestani et al. 2003).

MATERIAL & METHODS

Embryo Culture

Gametes were obtained from adult *S. purpuratus* by injection with 0.5 M KCl. The eggs were fertilized in filtered seawater containing penicillin at 20 U/ml and streptomycin at 50 μg/ml, and then incubated at 15 °C for the duration of development.

MEME Computational Analysis

Cis-regulatory sequences of five coexpressed differentiation genes (*Sp-Pks1, Sp-Fmo 1, 2, 3,* and *Sp-Sult*) from three sea urchin species (*S. purpuratus*, *M. franciscanus*, and *S. fragilis*) were aligned using Genboree Bioinformatics (www.genboree.org). The aligned sequences corresponded to the first intron, which includes a Sp-GCM DNA-binding site (Calestani and Rogers 2010). The aligned sequences were compared using multiple expectation maximization motif elicitation (MEME) analysis (Bailey and Elkan 1994). The MEME motifs 2, 13, and 30 were mapped on the -2Kb region and considered for further analysis.

Production of GFP Reporter Constructs

The *-2Kb-Gfp* control construct was previously prepared (Calestani and Rogers 2010). The *-2Kb* with either the mutated motifs 2, 13, or 30 were produced by de novo double-stranded oligonucleotide synthesis (Gen-Script USA Inc.). The de novo double-

stranded oligonucleotide had SacI and MluI sites at the 5' and 3' ends, respectively. Motif 2 was mutated from ACCACGCACTA to GAAGTATGAC. Motif 13 was mutated from TGGCACTACA to CAATGTCGTG. Motif 30 was mutated from GGGATTTCC to ATAGCGCAA. The *cis*-regulatory DNA mutagenized fragments were cloned into the EpGFPII reporter vector between SacI and MluI (Arnone et al. 1997). Each construct sequence was verified by restriction digestion and sequencing. Reporter constructs were linearized by SacI digestion and purified (QIAquick PCR purification kit, Qiagen) prior to embryo injection.

Embryo Injections

Embryo injection was performed as previously described (McMahon et al. 1985; Arnone et al. 2004). Injection solutions were prepared at a concentration of 1000 molecules/pl of linearized plasmid in 0.12 M KCl with the addition of 5× molar excess of restriction digested sea urchin genomic DNA (carrier DNA). Approximately 2 pl of injection solution was delivered to each embryo. Embryos injected with each GFP reporter construct were observed at the pluteus stage using fluorescence microscopy (Olympus BX60).

RESULTS

In order to identify additional *cis*-regulatory sequences in the *Sp-Pks1* promoter we used a comparative genomics approach. Specifically, we compared *cis*-regulatory regions of five coexpressed genes that are coregulated by GCM and GATAE, and hence are likely to belong to the same differentiation gene battery, *Sp-Pks1*, *Sp-Fmo 1*, *2*, and *3*, and *Sp-Sult* (Davidson 2006; Calestani and Rogers 2010). These five genes were compared in three different species of sea urchins, *S. purpuratus (SP)*, *M. franciscanus* (*MF)*, and *S. fragilis* (*SF*; Figure 1). The MEME computational analysis identified thirty conserved motifs (Table I) ranging from 11 to 14 bp.

We experimentally tested three motifs because they mapped close to the validated GCM, GATAE, and KRL-LIKE DNA-binding sites: motif 2, 13, and 30 (Table I; Figure 2). Motif 2 (*pksm2*) is localized at -1,227 bp of *Sp-Pks1*, 38 bp upstream of a GCM binding site, 153 bp downstream of a GATAE site, and 79 bp upstream of a KRL-LIKE site. Motif 13 (*pksm13*) is localized at -1,340 bp of *Sp-Pks1*, 151 bp upstream of a GCM binding site, 40 bp downstream of a GATAE site, and 192 bp upstream of a KRL-LIKE site. Motif 30 (*pksm30*) is localized at -561bp of *Sp-Pks1*, 610 bp downstream of a GCM binding site, 819 bp downstream of a GATAE site, and 568 bp downstream of a KRL-LIKE site.

The function of each motif was tested by mutagenesis of the *Sp-Pks1 -2Kb* region fused to the reporter *gfp*. All three mutagenized constructs showed a drastically reduced expression of *gfp*: while 44.83% of the embryos injected with the control construct showed expression in pigment cells only, 0%, 1.56%, and 2.8% of the embryos injected respectively with the *pksm2*, *pksm30*, and *pksm13* mutagenized constructs showed *gfp* expression in pigment cells only (Table II; Figure 3). These results indicate that the predicted *pksm2*, *pksm13*, and *pksm30* motifs contain DNA-binding sites for positive transcriptional regulators of *Sp-Pks1*.

Motif 2 in BLOCKS format

```
--------------------------------------------------------
MOTIF 2 width = 14 seqs = 15
SP_fmo1_P3          GCCGTGATAGGGGC
SP_fmo1_P1          GCCGTGATAGGGGC
MF_fmo1_P3P4        GCCGTGATAGGGGC
MF_fmo1_P1P2        GCCGTGATAGGGGC
SF_fmo1_P1P2        GCCGTGATAGGGGC
SP_fmo3_P1          GCGGGGTCAGCGGC
MF_fmo3_P1          GCGGGGTCAGCGGC
SF_fmo2_P1P2        GCGTGGACCGGGGC
MF_fmo2_P1P2        GCGTGGACXGGGGC
SP_fmo2_P2          GCGTGGCCCGGGGC
SP_fmo2_P1          GCCATGATAGAGGC
SF_fmo1_P3          GCCTGGGCGGCCGC
SP_sult_P1          GCCGTGTCAGACAC
SP_pks_P1           GCCGTGGTAGGCAT
MF_pks_P1           GCCGTGGTAGGCAT
```

Motif 13 in BLOCKS format

```
--------------------------------------------------------
MOTIF 13 width = 11 seqs = 15
SF_fmo1_P3          TCCGGGCACCG
SP_fmo3_P1          TCCTGGCACCG
MF_fmo3_P1          TCCTGGCACCG
SP_fmo2_P2          TCCGTGAACCG
MF_fmo2_P1P2        TCCGTGAACCG
SF_fmo2_P1P2        TCCGTGAACCG
SP_fmo1_P1          TGCGTGTACCG
SF_fmo1_P1P2        TGCGTGTACCG
SP_fmo1_P3          CCCGGGCACCG
MF_fmo3_P1P2        TCCTGGCACCA
SP_fmo2_P1          TGCCGGTACCG
SP_fmo1_P5          TCGTTGTACCG
MF_pks_P2           TGCGGGTACCA
SF_fmo1_P4P5        TCGTTGTACCG
MF_fmo1_P3P4        TCCTGGAACCX
```

Motif 30 in BLOCKS format

```
--------------------------------------------------------
MOTIF 30 width = 14 seqs = 2
SP_fmo1_P3          CGCTGGGCGATGCC
SF_fmo1_P3          CGCTGGGCGATGCC
```

Figure 1. MEME putative *cis*-regulatory sequence alignment of five coexpressed differentiation genes (*Sp-Pks1, Sp-Fmo 1, 2, 3,* and *Sp-Sult*) from three sea urchin species (*SP, MF,* and *SF*). Data shown are for the three motifs tested by mutagenesis.

Table 1. Comparative genomics analysis to identify conserved *cis*-regulatory motifs. List of the consensus motifs identified by the MEME analysis. The motifs that were tested in vivo by mutagenesis are highlighted in bold.

MEME Consensus Motif No.	Number of Aligned Sequences	Consensus Sequence (IUPAC)
1	18	HYCCCCSWSCCCCC
2	**15**	**GSYGKSAYMRGGGC**
3	28	CHKTYTAWRCATTT
4	10	CCMMWKTGCCCGTC
5	17	CDACHYTSTCCTCC
6	5	AYCCGTGTGCTTCG
7	21	ARTSSTCCGGC
8	28	TYTTCTYMKTTCTC
9	16	TRWWTSTYACTACA
10	28	TYKASTTYMCTTCA
11	10	CKKYBWWMCAGCCA
12	14	GMYSRACSAACGAA
13	**15**	**TSYDSGTACCG**
14	6	GCCAAGCCACTCAC
15	6	AWKCATACGTTTAG
16	5	TSGMTCGACGTTGA
17	12	AKRTTRRARAGAGA
18	9	TBKADGYGAGTATT
19	6	TYAWCSGAAAATGC
20	6	GKKSCKTGGTCGGT
21	5	GRKCTWCGCAGCAA
22	15	GHWGRVACTTCCCC
23	5	CATTCASGCAGGAA
24	6	GTTTYCACCCATC
25	18	MCMRTYAMCKTTAC
26	4	AAGTCTTAATTGAG
27	4	ACCYTYCTATATCG
28	2	GGCGGCGCCTGCTC
29	6	GKCSSGCGCCC
30	**2**	**CGCTGGGCGATGCC**

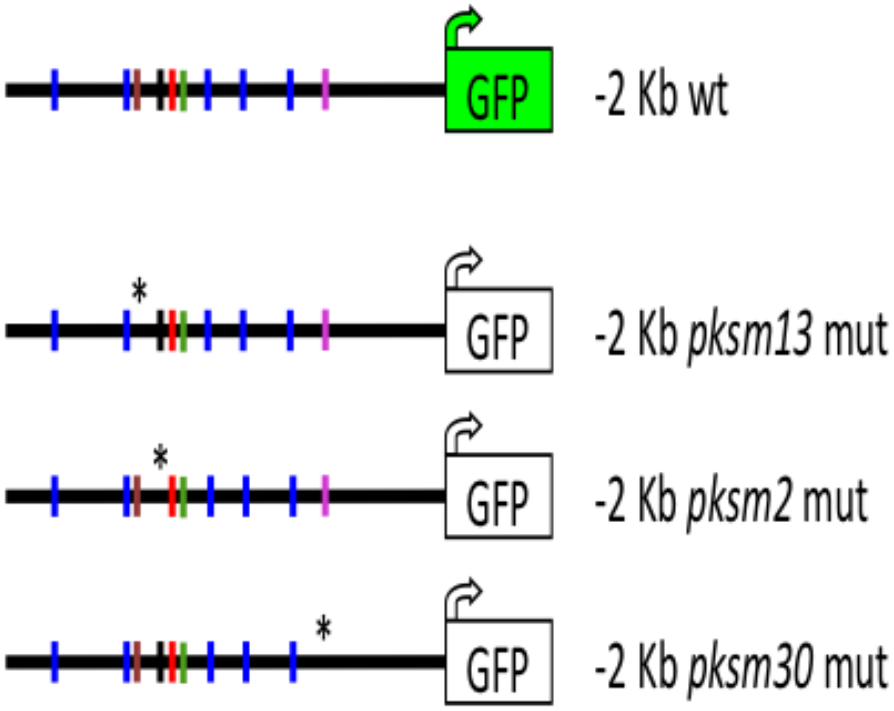

Figure 2. Mutagenesis of putative *cis*-regulatory motifs within the *-2Kb* of *Sp-Pks1*. Single motifs were mutagenized (indicated with *) and DNA constructs were injected into fertilized eggs. GFP expression was observed at the pluteus stage. GFP expression present in pigment cells is indicated by a green box. The GFP white box indicates no expression of the construct. Color-coded are the *cis*-regulatory motifs considered in this study, *pksm2* in black, *pksm13* in brown, and *pksm30* in pink. Previously functionally validated DNA-binding sites for GCM (red), KRL-LIKE (green), and GATAE (blue) are also indicated (Calestani and Rogers 2010).

Table II. *Cis*-regulatory activity of the *Sp-Pks1-Gfp* reporter constructs. Data reported were obtained from pluteus stage embryos. Each replicate experiment was performed on embryos produced from a different set of parents. Embryos were considered GFP positive if they had more than two fluorescent cells; GFP expression in only one or two cells was considered background due to the position of integration in the genome. The total number of scored embryos was obtained by combining all the replicate experiments.

Construct (# of Replicates)	% GFP Positive (# of Scored Embryos)	% GFP Positive Pigment Cells Only	% GFP Positive Ectopically
Control -2Kb (3)	48.28 (145)	44.83 (145)	3.45 (145)
pksm2 mutagenized (3)	0.70 (145)	0.00 (145)	0.70 (145)
pksm13 mutagenized (3)	7.70 (143)	2.80 (143)	4.9 (143)
pksm30 mutagenized (3)	2.34 (128)	1.56 (128)	0.78 (128)

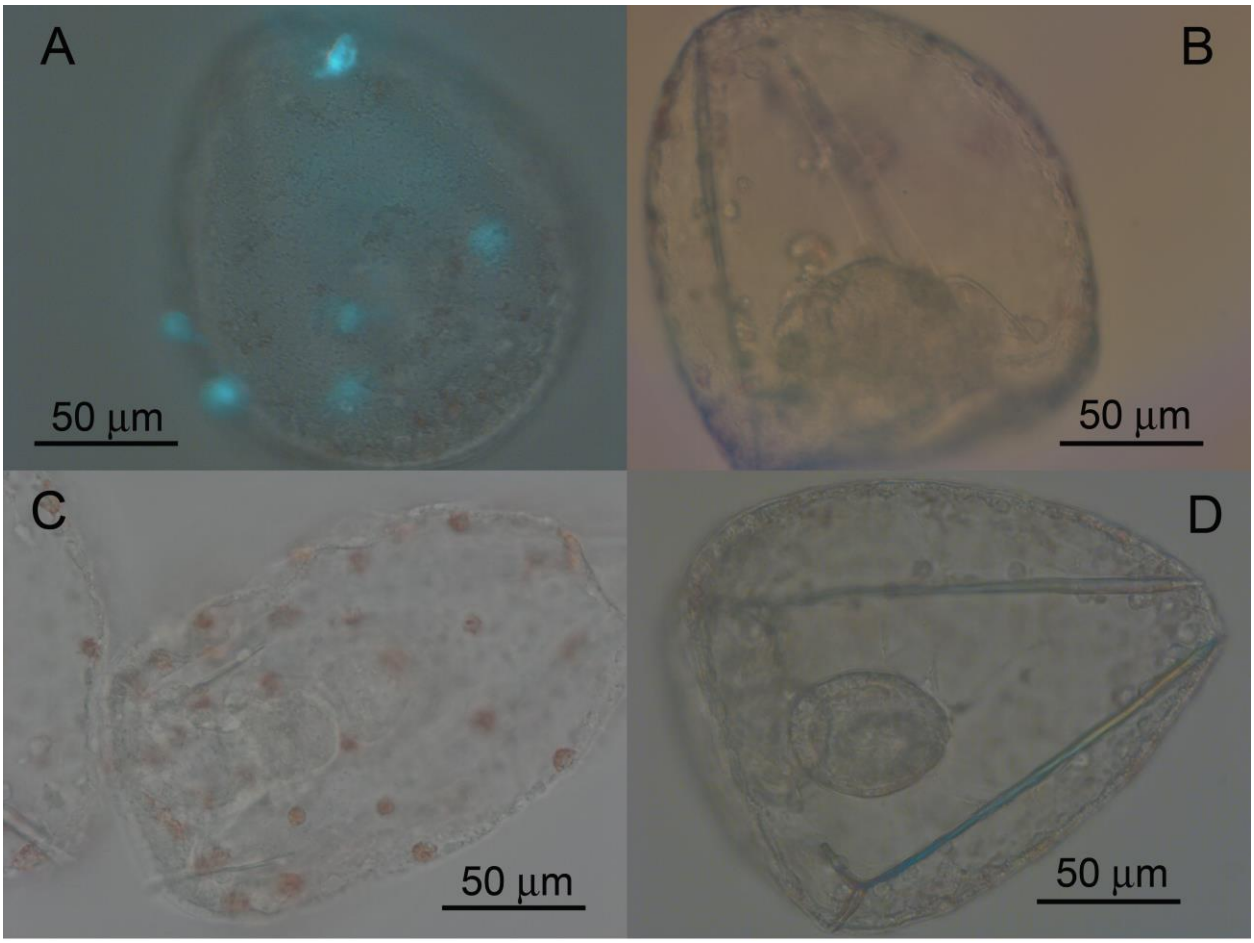

Figure 3. Mutagenesis of putative *cis*-regulatory motifs 2, 13, and 30. (A-D) Overlay of DIC and fluorescence images of embryos injected with the *-2Kb-Gfp* control construct (A), the *pksm2-Gfp* mutagenized construct (B), the *pksm30-Gfp* mutagenized construct (C), and the *pksm13-Gfp* mutagenized construct (D). GFP expression is observed in the control but not in the mutagenized construct injected embryos.

DISCUSSION

This study brings new insights into the GRN architecture required for NSM development and adds to the general knowledge of the structure and function of GRNs during the differentiation process. The use of comparative genomics involving the comparison of putative *cis*-regulatory sequences of five coexpressed and coregulated genes in three different sea urchin species was effective. Thirty conserved DNA motifs were identified (Table I). Three DNA motifs were tested for functionality by mutagenesis and all three include DNA-binding sites for positive regulators of *Sp-Pks1* (Table II; Figure 3). The *pksm2* and *pksm13* motifs mapped very close to previously validated DNA-binding sites for GCM, GATAE, and KRL-LIKE, in the range of 38–192 bp, which suggests that they belong to the same *cis*-regulatory module (Table I; Calestani and Rogers 2010; Davidson 2006). The *pksm13* motif is relatively close to the three previously validated DNA-binding sites but it might belong to an adjacent *cis*-regulatory module.

Previous studies demonstrated that the GRN for pigment cell development downstream of the D/N signaling is relatively shallow (Figure 4; Ransick et al. 2002; Calestani and Rogers 2010; Materna et al. 2013). The D/N signaling directly activates *Sp-Gcm*, which directly activates *Sp-Pks1*. This study, in combination with the study of Calestani and Rogers (2010), suggests that the expression of the *Sp-Pks1* differentiation gene is locked-down by multiple positive transcriptional regulators. Two of these positive *cis*-regulatory inputs are acting downstream of the 7th-9th cleavage D/N signaling through GCM and GATAE and one is D/N independent acting through KRL-LIKE (Ransick et al. 2002; Ransick and Davidson 2006; Calestani and Rogers 2010). It is not known if the rest of the direct regulators identified are D/N dependent or independent. It is possible that at least some are parallel positive regulatory inputs into differentiation genes as was observed for the SM development (Amore and Davidson 2006; Oliveri et al. 2008; Sun and Ettensohn 2014). Each positive transcriptional regulator is not redundant but is required for *Sp-Pks1* expression, resulting in an "all or nothing" transcriptional output. The "all or nothing" transcriptional output could be a mechanism that serves as a developmental check-point. Specifically, the *cis*-regulatory region of a differentiation gene, such as *Sp-Pks1,* might integrate the inputs of multiple genetic pathways that regulate earlier specification processes, and possibly coordinate pigment cell differentiation with the development of other embryonic territories.

Moreover, pigment cell specification and differentiation become independent from the D/N signaling through at least two positive feedback loops: one is produced by the activation of *Sp-Gcm* by itself, a second one is a triple positive feedback loop involving *Sp-Gcm*, *Sp-GataE*, and *Sp-Six1/2* (Figure 4; Ransick and Davidson 2006, 2012). Mathematical models of GRNs suggest that the presence of multiple interconnected positive feedback loops is a more effective mechanism for locking down the regulatory state of cells as opposed to single positive feedback loops (Hornung and Barkai 2008; Ben-Tabou de-Leon 2010; Ben-Tabou de-Leon 2016). Further robustness of the pigment cell GRN is provided by a feed forward loop involving *Sp-Gcm*, *Sp-GataE*, and *Sp-Pks1* (Figure 4; Ransick and Davidson 2006; Calestani and Rogers 2010; Materna et al. 2013).

Interestingly, as previously observed (Calestani and Rogers 2010), none of the deletion constructs produced a significant amount of ectopic expression of GFP. This suggests that direct repressors might not be required to restrict *Sp-Pks1* expression to pigment cells. Instead, previous studies have shown that the earlier process of specification sets the boundaries of the differentiation gene batteries' domain of expression (Levine and Davidson 2005; Oliveri et al. 2008; Solek et al. 2013; Materna et al. 2013). For example, *alx1* represses *gcm* in the SM at the blastula stage (Oliveri et al. 2008), *gcm* represses the blastocoelar cell fate in pigment cell precursors, and *not* represses *gcm* in the oral NSM (Solek et al. 2013; Materna et al. 2013). Indeed, *gcm* seems to be a key regulator of pigment cell development. In fact, its ectopic expression in SM cells is sufficient to develop a pigment cell's fate (Damle and Davidson 2012).

In conclusion, the transcriptional regulation of the pigment cell differentiation gene *Sp-Pks1* involves at least six positive *cis*-regulatory inputs. The GRN architecture upstream of *Sp-Pks1* is shallow and it includes multiple interconnected positive feedback loops, which contribute to lock-down its expression.

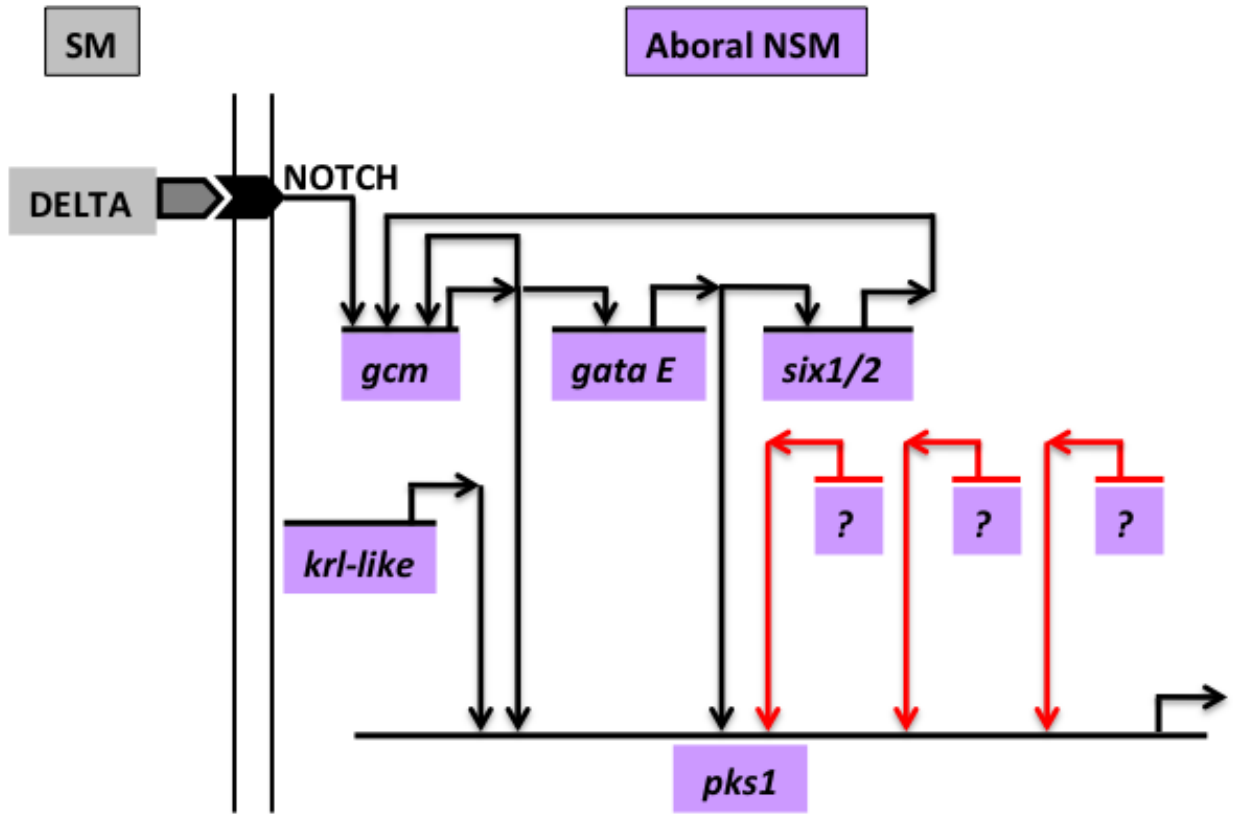

Figure 4. Graphic representation of the *S. purpuratus* pigment cell GRN upstream of *Sp-Pks1*. Arrows indicate positive regulatory inputs. Different embryonic territories are indicated and color-coded (SM and aboral NSM). The three positive regulatory inputs discovered in this study are indicated by red arrows, while the ones reported by previous studies are indicated in black (Ransick et al. 2002; Ransick and Davidson 2006; Calestani and Rogers 2010; Ransick and Davidson 2012; Materna et al. 2013).

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