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Array-Based Functional Screening of Heparin Glycans

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SUMMARY

Array methodologies have become powerful tools for interrogation of glycan-protein interactions but have critically lacked the ability to generate cell response data. Here, we report the development of a slide-based array method exemplified by measurement of activation of fibroblast growth factor signaling by heparin saccharides. Heparan sulfate-deficient Swiss 3T3 cells were overlaid onto an aminosilane-coated slide surface onto which heparin saccharides had been spotted and immobilized. The cells were transiently stimulated with FGF2 and immunofluorescence measured to assess downstream ERK1/2 phosphorylation. Activation of this signaling pathway response was restricted to cells exposed to heparin saccharides competent to activate FGF2 signaling. Differential activation of the overlaid cells by different-sized heparin saccharides was demonstrated by quantitative measurement of fluorescence intensity. This “glycobiarray” platform has significant potential as a generic tool for functional glycomics screening.

INTRODUCTION

Glycomics is an emerging field that aims to develop large-scale integrated systems approaches to study structure-function relationships of glycans. A review of the current state of the art in the field of glycomics reveals an emerging availability of high-throughput methods for interrogating structure-function relationships using glycan libraries (Otto et al., 2011). Microarrays have been the subject of considerable interest over recent years, as a powerful tool in the post-genome era for exploring gene expression and molecular interactions on a large scale. More recently, DNA and RNAi transfection, and small molecule screening, have been developed to monitor the responses of living cells that are overlaid onto microarray surfaces (Bailey et al., 2004; Lee et al., 2008; Wheeler et al., 2004). Microarrays displaying glycans—“glycoarrays”—for interrogation

with protein probes, developed by ourselves and others, are also proving critical for defining glycan-protein interactions, including heparin/heparan sulfate (HS)-protein interactions (Fukui et al., 2002; Blixt et al., 2004; Noti et al., 2006; Paulson et al., 2006; Zhi et al., 2006; de Paz et al., 2007; Laurent et al., 2008; Wakao et al., 2008; Zhi et al., 2008; Song et al., 2011a, 2011b). In relation to cell interactions, glycan arrays have been applied to the assessment of adhesion of bacteria, viruses, and mammalian cells to glycans (Nimrichter et al., 2004; Dickinson et al., 2010; Šardžik et al., 2011; Song et al., 2011a, 2011b; Arndt et al., 2011). However, to the best of our knowledge, their application to generate functional data on the consequences of cell-glycan interactions in terms of cellular responses of living cells (such as activation of intracellular signaling pathways) has not been demonstrated.

HS is a ubiquitous linear glycosaminoglycan that is found attached to any of several families of core proteins to form complex glycoproteins. It is diverse in its cellular and extracellular distributions and is critical in a range of biological activities from development to signaling regulation (Bernfield et al., 1999). A highly sulfated version of HS, heparin, is used as a pharmaceutical drug for its anticoagulative properties. The structural diversity of HS within and between tissues is generated by the action of a complex family of biosynthetic enzymes. Spatial and temporal regulations of the expression and bioactivity of the enzymes, as well as the fact that the reactions are not template driven and do not go to completion, account for the diversity in HS structure (Turnbull et al., 2001). These modifications occur in a regulated fashion with certain modifications occurring in discrete areas. Regions of modifications tend to cluster in highly sulfated areas (S domains), whereas stretches on unmodified disaccharides containing N-acetylated glucosamine residues form NA domains (Turnbull and Gallagher, 1990; Turnbull et al., 2001). One of the major challenges of the field is to analyze the biological activity of the many different species of HS in order to develop a detailed understanding of their structure-function relationships (Powell et al., 2004).

Here, we describe a novel slide-based assay, which, to our knowledge, provides for the first time (Figure 1) a functional glycoarray format. It relies on measurement of responses by fluorescence detection of endogenous markers, and avoids reliance on sophisticated microscopic equipment for image acquisition. We demonstrate proof of concept and a key application by

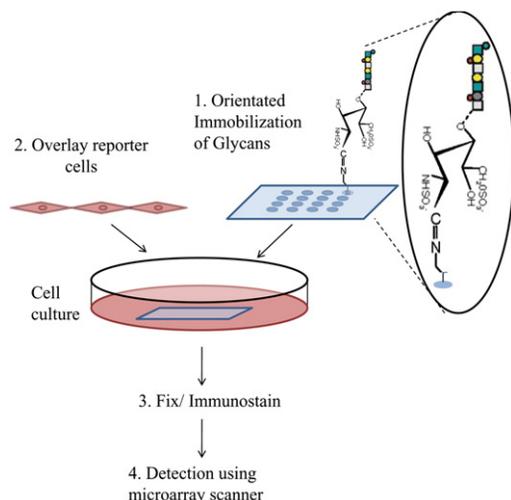


Figure 1. A Slide Array Method for Assaying Functional Responses of Cells to Immobilized Saccharides

Saccharides can be immobilized onto an aminosilane glass surface via a Schiff's base linkage with their reducing ends, as shown in the inset. Reporter cells are then overlaid onto the slide surface and cultured for a specified period, followed by fixation and immunostaining to detect specific epitopes using a microarray slide scanner.

See also Figure S1.

demonstrating that the methodology can be used to screen prototypical activation of fibroblast growth factor (FGF) signaling by heparin saccharides with differential activities. This assay platform allows the simultaneous functional assessment of different HS/heparin structures and should have broad utility as a new glycomics tool.

RESULTS

Slide-Based Assay for FGF Signaling Responses

To demonstrate proof of concept of a slide-based assay, we chose to study activation of FGF signaling by HS in sulfate-deficient Swiss 3T3 cells. These have the advantages that they naturally express only a single isoform of FGF receptor, FGF receptor 1 IIIc (FGFR1IIIc) (Pelech et al., 1986), and that the procedure for the depletion of HS sulfation using chlorate is well characterized (Rapraeger et al., 1994). Thus, responses to FGF can be directly ascribed to exogenous addition of saccharides and signaling by FGFR1IIIc. The efficacy of the chlorate-induced depletion of HS sulfation in Swiss 3T3 cells grown on aminosilane-coated surfaces was confirmed by immunofluorescence using an anti-HS antibody that recognizes sulfated epitopes (see Supplemental Experimental Procedures and Figure S1A available online). We chose to examine signaling responses to FGF2 via phosphorylation of two important members of the mitogen-activated protein kinase (MAPK) family pathway: the extracellular signal-regulated kinases 1 and 2 (ERK1/2) (Cano and Mahadevan, 1995). Because ERK1/2 activation in Swiss 3T3 cells has been shown to be dependent on cell adhesion (Pennington et al., 2007), and can alter responses to growth factors (Sergeant et al., 2000), we first determined whether adhesion of chlorate-treated cells was similar on aminosilane slides and standard

tissue culture glass substratum; immunofluorescence for β -actin and vinculin confirmed that cell adhesion was similar on these surfaces (Figure S1B).

Next, we examined a variety of spotting conditions to optimize saccharide coupling via Schiff's base formation (Yates et al., 2003). Heparin 12-mer saccharides, which are known to activate FGF signaling (Delehedde et al., 2000), were spotted in triplicate in different solutions (500 nL each) onto an aminosilane surface and coupled via a microwave-enhanced reaction (Powell et al., 2009). Chlorate-treated cells were overlaid onto the slide and following serum starvation were treated with FGF2 (10 ng/mL) for 90 min. Cells were then fixed and stained for both total and phosphorylated ERK1/2 (pERK1/2), using specific secondary antibodies conjugated to different fluorophores, corresponding to the two wavelengths detected by a microarray scanner (535/557–592 nm and 635/650–690 nm) (see Experimental Procedures). Fluorescence slide scanning demonstrated that betaine produced spots with the most significantly increased levels of fluorescence intensity for pERK1/2 (Figures 2A–2C, S2A, and S2B), with no significant change in total ERK1/2 (Figures 2D, 2E, and S2C). A signal-to-noise ratio of \sim 7:1 was observed for pERK1/2 at 90 min (Figure 2C), indicative of sustained activation of FGF signaling observed in previous experiments in standard cell culture (Delehedde et al., 2000). In contrast the other spotting conditions did not produce significantly increased pERK1/2 (Figure S2B), and levels of total ERK1/2 were unaltered by the different sugar-conjugation reaction conditions (Figure S2C). Betaine is also well established for improving spot homogeneity in microarrays (Diehl et al., 2001) and was chosen for all further experiments.

Inhibition of ERK1/2 Activation in Slide-Based Assay

To confirm the specificity of the immunofluorescence pERK1/2 detection of FGF signaling, we employed the inhibitor PD098059 that inhibits phosphorylation of pERK1/2 (Delehedde et al., 2000) by binding to the inactive forms of MEK1 and preventing activation by upstream activators such as c-Raf. A total of 50 μ M PD098059 effectively inhibited levels of DNA synthesis in chlorate-treated Swiss 3T3 cells in standard culture conditions, using western blotting to analyze phosphorylated and total ERK protein levels (Figure S3A). Addition of the inhibitor reduced the level of pERK1/2 down to negative control levels. No effect was observed on phosphorylation with the addition of inhibitor in the absence of FGF2 stimulation. Pretreatment with the inhibitor PD098059 prior to FGF2 and heparin 12-mer saccharide stimulation was also associated with inhibition of DNA synthesis levels (Figure S3B), a commonly used measure of cell proliferation. When MEK1 inhibitor was added to the medium of chlorate-treated cells cultured on aminosilane slides prior to the addition of FGF2, elevation of pERK1/2 in the presence of heparin 12-mer saccharide was abolished (Figure S3C). Thus, the immunofluorescence observed by slide scanning depends specifically on phosphorylation of ERK1/2 induced by activation of FGF signaling.

Measurement of Differential Activation of FGF Signaling

We next investigated whether the slide-based assay can be used as a tool for screening the differential activities of saccharides, using three size-defined heparin saccharides predominantly

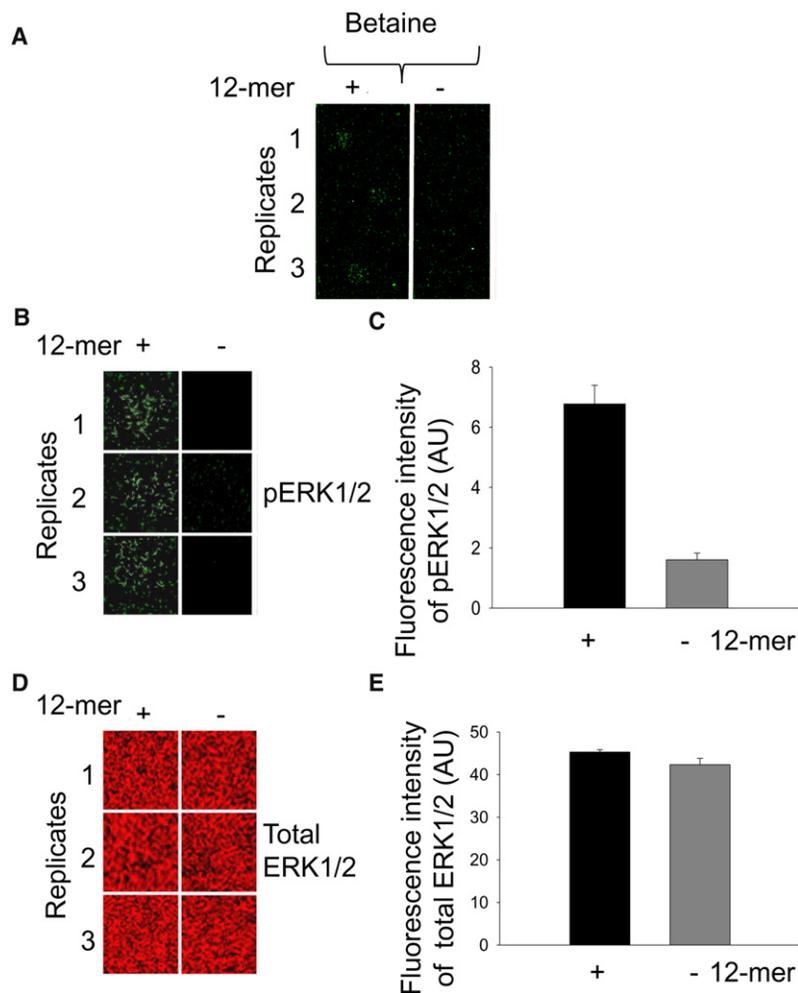


Figure 2. Immobilized Heparin Oligosaccharides Induce ERK1/2 Phosphorylation in HS-Deficient Cells Responding to FGF2 in a Slide-Based Assay

(A) An aminosilane slide was spotted with a heparin 12-mer saccharide or control (no saccharide) in betaine, and overlaid with HS-deficient (chlorate-treated) Swiss 3T3 cells. After overnight adherence and 24 hr serum starvation, the cells were stimulated with FGF2 for 90 min and then fixed and stained for pERK1/2 using anti-p44/42 primary antibody and anti-mouse Alexa Fluor 555 secondary antibody (see Experimental Procedures).

(B) and (D) show individual triplicate spots immunostained for pERK1/2 or total ERK1/2 protein, respectively.

(C) and (E) show fluorescence intensity of spot areas for pERK1/2 and total ERK1/2, respectively, measured using a slide scanner (mean \pm SD for triplicate spots from a single slide). AU, arbitrary units.

See also Figure S2.

Quantitative analysis of spot intensity demonstrated statistically significant activation of signaling (measured by elevated pERK1/2) by both 12- and 18-mer heparin saccharides, whereas the 2-mer was not significantly different from the negative control (Figure 3D), and corresponding immunofluorescence microscopy images in Figure S3F), whereas total ERK1/2 levels were not significantly altered (Figure 3E). These data are in agreement with data from standard cell culture (Figure S3E) and previous literature data (Delehedde et al., 2002). Differential activation was also evident because a statistically significant higher level of ERK phosphorylation to produce pERK1/2 was noted for the 18-mer compared to the 12-mer saccharide (Figure 3D;

composed of standard repeating trisulfated disaccharide units (for preparation details see Supplemental Experimental Procedures and Figure S3D). These comprised two activating heparin saccharides (12- and 18-mer) and a nonactivating counterpart (2-mer), previously tested in cell culture assays (Delehedde et al., 2000; Walker et al., 1994). Saccharides were spotted and responses to FGF2 measured as described above for Figure 2. Analysis of the array demonstrated increased immunofluorescence for pERK1/2 for the heparin 12- and 18-mer spots (Figures 3A and 3B). In the case of the heparin 2-mer and negative control, there was no increase in fluorescence intensity when compared to the surrounding areas. Total ERK1/2 was comparable across the different conditions (Figure 3C). The activity of these saccharides was also consistent with DNA synthesis assays with chlorate-treated Swiss 3T3 cells in standard cell culture (Figure S3E). In chlorate-treated cells FGF2 alone or in the presence of disaccharides did not stimulate DNA synthesis and only elicited an early transient dual phosphorylation of p42/44 MAPK, whereas FGF2 in the presence of tetrasaccharides and longer oligosaccharides was able to restore DNA synthesis and enable the sustained dual phosphorylation of p42/44MAPK (Delehedde et al., 2000).

$p = 0.04$). Differential cell responses to spotting concentration were observed to be dose dependent in this glycobiarray format (Supplemental Experimental Procedures; Figure S3G). This is consistent with previous cell culture literature, and suggests as expected a dependence on the amount of glycan immobilized; the latter varies with spotting concentration, as noted previously for measurement of protein binding in standard glycoarrays (Zhi et al., 2006; Noti et al., 2006; de Paz et al., 2007; Zhi et al., 2008; Powell et al., 2009). Overall, these data provide evidence that this technique is sufficiently sensitive to measure quantitative differences in cell responses to immobilized heparin glycans in terms of FGF2 signaling activation via detection of the levels of pERK1/2 in the MAPK pathway.

DISCUSSION

Here, we have demonstrated a method for measuring the responses of living cells overlaid on immobilized glycans in a slide-based array format (Figure 1). The activation of FGF2 by the heparin/HS family of glycans provides a prototypic system for demonstrating this assay. HS-deficient (chlorate-treated) Swiss 3T3 cells were overlaid onto aminosilane-coated slide surfaces onto which heparin saccharides had previously been

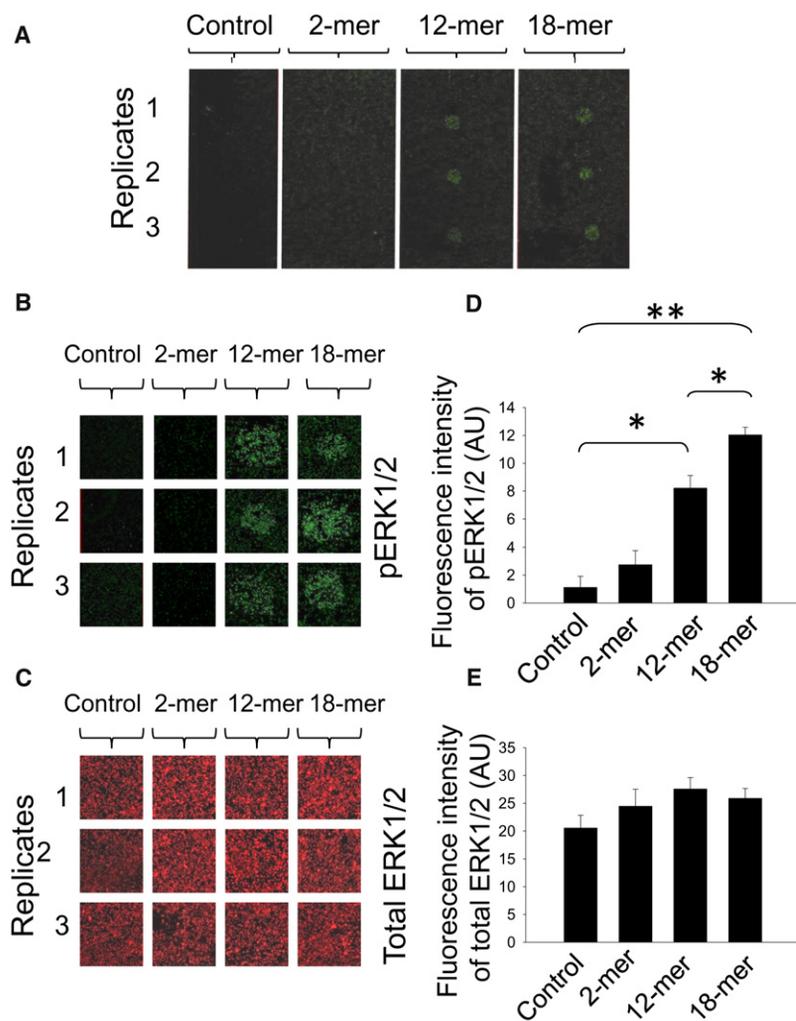


Figure 3. Slide-Based Assay of FGF Signaling Activation by Size-Defined Heparin Oligosaccharides

(A) HS-deficient (chlorate-treated) cells were seeded onto an aminosilane slide spotted with various saccharides in betaine and treated as described in Figure 2 to measure FGF2 signaling, and array was immunostained for pERK1/2.

(B) and (C) show individual triplicate spots immunostained for pERK1/2 or total ERK1/2 protein, respectively.

(D) and (E) show fluorescence intensity of spot areas for pERK1/2 and total ERK1/2, respectively, measured using a slide scanner (mean \pm SD for triplicate spots from a single slide). Statistical analysis was performed using the two-tailed t test assuming equal variance: ** $p \leq 0.005$ and * $p \leq 0.05$ ($n = 3$). In (D) the 2-mer heparin saccharide and negative control are not significantly different. AU, arbitrary units. See also Figure S3.

measuring binding of bacteria, viruses, and mammalian cells to immobilized glycans (Nimrichter et al., 2004; Dickinson et al., 2010; Šardžik et al., 2011; Song et al., 2011a, 2011b; Arndt et al., 2011), to our knowledge, this is the first demonstration of a slide-based technology for analysis of cellular responses to glycans. Current in vitro functional assays using cell biology approaches often require high cell numbers or larger amounts of reagents that are not always available, especially in the case of saccharides. Therefore, the development of slide-based functional screening assays may allow the screening and differentiation of different and larger populations of saccharide structures. The platform we describe here has significant potential for further development as a generic tool for functional glycomics screening of a wide range of glycans. The meth-

odology is easily applicable to a standard laboratory setting because images are acquired using only a DNA microarray scanner. It has a number of advantages, including rapidity, provision of miniaturization (and thus low reagent usage), and also significant potential for development as a high-throughput tool.

SIGNIFICANCE

The development of array-based assay technologies is a much-needed method for assessing glycan function, especially in the case of the heparan sulfate (HS) family. This methodology now has the potential to be extended to study natural HS saccharide libraries, the production of which has recently been described by Powell et al. (2010), and also HS saccharides produced by synthetic chemistry (Noti et al., 2006; de Paz et al., 2007; Arungundram et al., 2009). Furthermore, such saccharides can be robotically spotted into array formats (Zhi et al., 2006, 2008; Noti et al., 2006; de Paz et al., 2007; Powell et al., 2009) that would allow the large-scale simultaneous analysis of different structures for the first time. Such studies could directly address the structure-function relationships of HS saccharides at

spotted and immobilized. The cells were transiently stimulated with FGF2 and immunofluorescence measured to assess the level of ERK1/2 phosphorylation. Activation of this signaling pathway response was restricted to cells exposed to heparin saccharides competent to activate FGF2 signaling. Differential activation of the overlaid cells by different-sized heparin saccharides was demonstrated by quantitative measurements of fluorescence intensity, and was consistent with previously published data in traditional cell assays.

This slide-based functional screening assay can be used to simultaneously screen different populations of heparin/HS saccharide structures. The screening tool obtains quantitative data from facile fluorescence intensity measurements, producing clear differentiation of different species of HS/heparin. The immunostaining protocol used here for analyzing the activation of pERK can be readily adapted by changing the choice of antibody used in the protocol; thus, a range of different cell markers can be assessed to study different cell processes such as cell differentiation and cell growth.

Although array methods have been described employing living cells for RNAi transfection and small molecule responses (Bailey et al., 2004; Erfle et al., 2007; Wheeler et al., 2004, 2005), and

a functional level, and might be further exploited as a generic tool for glycan analysis.

EXPERIMENTAL PROCEDURES

Cell Culture and Depletion of Sulfation from Swiss 3T3 Cells

Swiss 3T3 cells (ATCC CCL-92) were grown in a medium depleted of sulfate and containing sodium chlorate to inhibit the sulfation of endogenous HS proteoglycans. The sulfate-free culture was prepared without sodium chloride or cystine using individual ingredients devoid of sulfate also known as the sulfate-free medium. A detailed explanation of reagents and methods is given in Rapraeger et al. (1994). Different conditions were used as controls to block or restore the inhibition of HS sulfation, including pretreatment medium A and B, as previously described by Rapraeger et al. (1994).

Immobilization of Oligosaccharides onto Aminosilane Surfaces

The preparation of size-defined heparin oligosaccharides using heparinase digestion and gel filtration chromatography (Powell et al., 2010) is described in Supplemental Experimental Procedures and Figure S3. Freeze-dried oligosaccharides (at final concentration 1 mM) were dissolved in betaine (Sigma-Aldrich), betaine and formamide (Fluka), water, or formamide. Betaine was used at 1.5 M. Spots were grouped in an appropriate area of the aminosilane glass slide, which was marked using a diamond-tipped pen. Sugars were spotted manually by pipette in triplicate (500 nl per spot), and the slide was microwaved and prepared as previously described by Powell et al. (2009). Molarity was used instead of weight in order to provide an equal comparison between different-sized heparin saccharides.

Optimization of Spotting Conditions

Cells were chlorate treated as previously explained. Cells were washed with PBS, split using 0.5% TE, and allowed to adhere for 24 hr onto the slide in a 10 cm sterile tissue culture dish (Corning). Cells were then washed and serum starved for 24 hr in pretreatment media A containing 0.1% (w/v) dialyzed BSA. Cells were incubated in pretreatment media A for 24 hr at 37°C. Cells were stimulated with 10 ng/ml FGF2 for 90 min at 37°C and 5% CO₂. After 90 min the cells were washed with ice-cold PBS and fixed with 4% paraformaldehyde. The cells were washed with PBS, blocked with 5% BSA (w/v) in PBSTX. Anti-phospho-MAPK IgG antibody primary antibody phosphorylated-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) antibody (Cell Signaling) primary antibody was added in 3% BSA in PBSTX and used at 1/200 dilution with overnight incubation at 4°C. The slide was washed three times in PBS and the secondary Alexa Fluor 555 anti-mouse IgG Fab fragment antibody (excitation 555 and emission 565). Total ERK was measured using p44/42 MAPK (ERK1/2) antibody (Cell Signaling), and anti-rabbit Alexa Fluor 635 was used as a secondary antibody. The slide was washed in PBS, allowed to dry, and stored in a fluorescence case until scanned. Inhibitors of MEK1 (in solution PD98059 [Calbiochem]) were added as detailed in the figure legends.

Microarray Scanning

Scanned images were obtained using an AXON GenePix 4000A (Axon Instruments). Axon voltage and signal intensities were set to 100%, respectively, of 635 and 535 nm wavelength. Mean signal intensities were calculated for the total signal in the spot area. ImageJ software was used for image acquisition and analysis.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.chembiol.2012.03.011.

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