Distinct gene expression profiles and reduced JNK signaling in retinitis pigmentosa caused by RP1 mutations

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To understand the mechanisms underlying autosomal dominant progressive retinitis pigmentosa (RP) caused by the mutations of the RP1 gene and to identify molecules that play roles in the early disease process, we used Affymetrix U74Av2 microarrays to compare the gene expression profiles of retinas from Rp12/2 and Rp11/1 mice at postnatal days (P) 7, 10, 14, 18 and 21. These profiles were independently verified by comparison with results of retinal serial analysis of gene expression, U74Av2 array studies of mouse retinas, real-time PCR and in situ hybridization. We found that the disruption of Rp1 significantly affected the expression of multiple clusters of genes whose products were involved in diverse biological pathways. The molecular responses to the disruption of Rp1 changed dramatically during development and were distinct from responses to the disruption of photoreceptor transcription factors (Crx−/− or Nrl−/−) and a photo-transduction molecule (Pde6brd1). We found specific alterations of gene expression in the c-Jun N-terminal kinase (JNK) signaling cascades. Western analysis confirmed that the phosphorylation of key members in the JNK signaling cascades (i.e. JNK1, JNK2, MAP2, MKK4 and c-Jun) is reduced, whereas phospho-ERK and phospho-p38 are unchanged, in Rp1−/− retinas at P18–21. Immunostaining demonstrated that, like Rp1, phospho-JNKs and phospho-MAP2 are present in outer segments of photoreceptors. Our studies reveal unique molecular phenotypes in multiple biological pathways and the specific reduction of JNK signaling cascades in RP1 diseases, and suggest that RP1, a doublecortin-containing microtubule associated protein, and JNK signaling cascades play integral roles in photoreceptor development and maintenance. Our studies further suggest JNK-related therapeutic strategies for RP1 diseases.

INTRODUCTION

Mutations in the gene encoding the retinitis pigmentosa 1 (RP1) protein account for 5–10% of cases of autosomal dominant, progressive RP in humans (1–4). The RP1 gene encodes a protein of 2156 amino acids whose N-terminal region has significant homology to that of human doublecortin (DCX), mutation of which causes abnormalities in the cerebral cortex (5,6). The mouse Rp1 gene is specifically expressed in photoreceptors during postnatal development, and the Rp1 protein, which begins to be detectable at P3–5, localizes to the outer segment (OS) axoneme of rods and cones (7,8).

We reported that a targeted disruption of Rp1 results in progressive degeneration of photoreceptors (9). In Rp1−/− retinas, OS disks were morphologically abnormal and of aberrant size as early as P7 when the OS had just begun to develop. Recently, another independent allele of Rp1 in which a truncated N-terminal Rp1 was created in mice displayed similar defects in morphogenesis of the photoreceptor OS (10). These findings indicate that Rp1 is required for the morphogenesis of the OS of photoreceptors. Furthermore, in our Rp1−/− mice, rhodopsin was mislocalized in photoreceptors, indicating that Rp1 might also be involved in the transport of proteins from the inner segment to the OS in photoreceptors (9).
Electrophysiologically, both Rp1 mutant retinas maintained normal kinetics of phototransduction but the amplitudes of their electroretinograms were progressively reduced (9,10). Further analysis of the Rp1 mutant retinas revealed that Rp1 binds to microtubules through its N-terminus containing the DCX domain, a result consistent with the role of DCX (11,12).

The c-Jun N-terminal kinases (JNKs) are a subfamily of the mitogen-activated protein kinases (MAPks) (13). In mammals, JNKs are encoded by three-related genes (14,15): JNK1 and JNK2 are expressed ubiquitously during development, whereas JNK3 is restricted to brain, heart and testis. JNK signaling is regulated by three-tiered phosphorylation cascades composed of MAPK, MAPKK (MAPK kinase) and MAPKKK (MAPK kinase kinase); JNKs phosphorylate their downstream targets such as microtubule-associated protein 2 (MAP2) which regulates cytoskeletal dynamics, and c-Jun which further regulates the transcription of other target genes. JNK signaling cascades have been implicated in retinal pigment epithelium, retinal ganglion cells and other cell types in the inner nuclear layer under ischemia, optic nerve transection or glaucoma conditions (16–20). Activating protein 1 (AP-1), which is a protein complex and substrate of JNKs, might be involved in the retinal degeneration in the rd mouse model (21). However, the role of JNK signaling cascades in photoreceptors in normal development and disease conditions remain largely unknown.

In the present study, we identified distinct genome-wide molecular phenotypes in Rp1−/− retinas by using microarray technology. We provide evidence that the JNK signaling cascades are specifically compromised in Rp1−/− retinas and that Rp1 and JNK cascades play integral roles in photoreceptor development and maintenance.

RESULTS

General assessment of Affymetrix microarray results

We focused on the early postnatal development of retinas when Rp1 was newly present in photoreceptors in Rp1+/+ mice and when the early abnormalities of photoreceptors were first observed and no significant photoreceptor cell loss was detected in Rp1−/− mice (9). Although Rp1 is not a transcription factor, the transcriptional responses of other genes to the disruption of Rp1 in Rp1−/− retinas could provide evidence for the involvement of the products of these genes in normal Rp1 function.

We used mice of F3–F4 generations of mixed background that were the offspring of intercrosses between 129SvEv and C57Bl/6 mice (9). To minimize strain-associated and sex-related variations, we pooled RNA from the neural retinas of 3 or 4 mice of each age and genotype. Genechip hybridizations were performed in triplicate for each age and genotype. To minimize variations associated with light responses, we collected retinas between 1 and 2 p.m. each day from mice kept on a light cycle of 6 a.m. to 6 p.m.

Original results of 30 Affymetrix array hybridizations have been deposited in the database (GEO series numbers: 117–120, 122–129; www.ncbi.nlm.nih.gov/geo/). We analyzed the 30 hybridizations following Affymetrix standards and discarded one of the P21 Rp1−/− samples due to its poor quality. For the remaining 29 hybridizations, the average scaling factor (the number by which the average intensity of signal value of every array is adjusted to a common value to make the arrays comparable) was 18.12 ± 1.60 (mean ± SEM). The 3’-to-5’ ratio for GAPDH was 0.85 ± 0.01 (mean ± SEM). These figures suggest the hybridizations are comparable to each other and there was no degradation or inefficient transcription of cDNA or biotinylated cRNA. We evaluated variation among three independent hybridizations of RNA from animals of each age and genotype by determining the numbers of genes detected on the array (Affymetrix Microarray Suite 5.0). The numbers of genes detected differed minimally between the two genotypes for mice of each age and among mice of the same genotype but of different ages. These results verify the reliability of our procedures and show that ~42% of probe sets in the U74Av2 array detect genes in the retina, consistent with the previous result (22).

Analyses of Affymetrix data from Rp1+/+ and Rp1−/− mice

We performed the following three different types of statistical analyses of our Affymetrix data from Rp1+/+ and −/− retinas (Table 1).

Analysis I: Identification of genes whose expression changes during retinal development (from P7 to P21) in Rp1+/+ mice. At a significance level of P ≤ 0.001 (one-factor ANOVA), 355 genes had expression patterns that changed between P7 and P21 (Supplementary Material, Table S1).

Analysis II: Identification of genes that are expressed differentially in Rp1+/+ and −/− retinas at each time point. Approximately 100 genes were expressed at significantly different levels in Rp1+/+ and −/− retinas at each time point (Student’s t-test, P ≤ 0.01; Supplementary Material, Table S2).

Analysis III: Identification of genes whose expression during development was affected by the targeted disruption of Rp1. We used two-factor ANOVA to identify 340 genes whose expression level differed significantly over five time points and two genotypes with a statistical significance α < 0.05 (Table 1) and 74 genes with α < 0.01 (Supplementary Material, Table S3).

Because of the large number of total probe sets we analyzed (12 488 on each chip), we estimated the False Discovery Rate (FDR) for each analysis (23). The optimal significance (α) levels by the profile information criterion and the corresponding FDR are shown in Supplementary Material, Table S4. Most of the α levels were close to 0.01, which we chose as a general cut-off value. Analysis I yielded an estimated FDR of ~0.4%, whereas analyses II and III had FDRs of 72.51%, 74.37%, 73.27%, 73.11%, 69.86% and 73.55%, respectively (Supplementary Material, Table S4). Although strikingly high, these estimates involving comparisons of wild-type and mutant retinas are extremely conservative and dependent on individual and overall variations among these 29 hybridizations as well as the small number (3) of replicates for each genotype and time point. These high estimates in
analyses II and III prompted us to further verify these microarrays results using independent methods.

### Verification of Affymetrix microarrays results on Rpl1<sup>+/+</sup> and Rpl1<sup>-/-</sup> retinas

Comparison of our results with results of SAGE and other Affymetrix analyses in Rpl1<sup>+/+</sup> retinas. To provide verification of our Affymetrix results, we first compared our results at P7, P10 and P21 with those provided in a comprehensive analysis of serial analysis of gene expression (SAGE) libraries from Crx<sup>+/+</sup> retinas at comparable ages (P6.5, P10.5 and adult) (24). We compared genes identified in our Affymetrix analysis I (355 genes between P7 and P21 in Rpl1<sup>+/+</sup> retinas) to genes identified as photoreceptor-specific/enriched or present in outer nuclear layer libraries in Rpl1<sup>+/+</sup> retinas and a total of 67 genes are common. Because Affymetrix signals are not directly compared with values given in SAGE libraries (percentage of clone representation), for each of the 67 genes we calculated the ratios between the Affymetrix signals obtained at two developmental time points (P10:P7 and P21:P10) and between the values given in SAGE at similar time points (P10.5:P6.5 and adult:P10.5). A total of 92 of 134 comparisons (two comparisons for each gene; 68.7%) are consistent between our Affymetrix and SAGE analyses. For 23 photoreceptor-specific/enriched genes among the 67 genes identified, 37 (80.4%) of the 46 comparisons displayed consistent changes between time points in the same direction (Table 2). We thus conclude that Affymetrix analysis and SAGE of Rpl1<sup>+/+</sup> retinas produce comparable results and are positively correlated, particularly for photoreceptor-specific genes.

Recently, a comprehensive gene expression analysis of mouse postnatal retinas using U74Av2 arrays has been reported (22). Therefore, we compared our results with those at comparable ages (at P10, P14 and P21), despite that the BALB/c strain was used in their study. We again focused on fold changes between P14 and P10, P21 and P14 for each of the common genes identified in both studies. Among the 355 genes identified in our analysis I and 710 corresponding fold changes, 87% showed changes in the same direction. After removal of the genes that are called as ‘absent’ in either data set, quantitative correlations of the remaining 532-fold changes between these two data sets were significant (R² = 0.66) (Fig. 1).

### Table 1. Numbers of genes identified by the three types of analyses of Affymetrix data from Rpl1<sup>+/+</sup> and Rpl1<sup>-/-</sup> retinas

<table>
<thead>
<tr>
<th>Time point</th>
<th>Number of genes identified&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>P7–P21</td>
<td>355</td>
<td>I (temporal pattern in Rpl1&lt;sup&gt;+/+&lt;/sup&gt;)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>P7</td>
<td>101</td>
<td></td>
</tr>
<tr>
<td>P10</td>
<td>93</td>
<td></td>
</tr>
<tr>
<td>P14</td>
<td>86</td>
<td></td>
</tr>
<tr>
<td>P18</td>
<td>88</td>
<td></td>
</tr>
<tr>
<td>P21</td>
<td>56</td>
<td>II (Rpl1&lt;sup&gt;+/+&lt;/sup&gt; versus Rpl1&lt;sup&gt;-/-&lt;/sup&gt; at each time point)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>P7–P21</td>
<td>340</td>
<td>III (interaction between genotype and time point)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>For analyses I, α = 0.001; for analyses II, α = 0.01; for analysis III, α = 0.05.

<sup>b</sup>Two-sample Student’s t-test.

### Table 2. Comparison of results of SAGE and Affymetrix analysis of changes in the expression of photoreceptor-specific genes in Crx<sup>+/+</sup> and Rpl1<sup>+/+</sup> retinas, respectively, at three developmental time points

<table>
<thead>
<tr>
<th>Gene</th>
<th>Affymetrix</th>
<th>SAGE</th>
<th>P10/P7</th>
<th>P21/P10</th>
<th>P10.5/P6.5</th>
<th>Adult/P10.5</th>
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<tr>
<td>Cdr2</td>
<td>2.2</td>
<td>7.7</td>
<td>7.4</td>
<td>0.0</td>
<td>1.2</td>
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<tr>
<td>Cga1</td>
<td>2.6</td>
<td>3</td>
<td>2.3</td>
<td>3.5</td>
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<td></td>
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<tr>
<td>D6Wsl76e</td>
<td>1.4</td>
<td>3.2</td>
<td>1.5</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dpl1/1</td>
<td>2.8</td>
<td>2.7</td>
<td>1.9</td>
<td>3.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gnat1</td>
<td>7.7</td>
<td>11.7</td>
<td>27.5</td>
<td>0</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Gnat2</td>
<td>1.1</td>
<td>2.5</td>
<td>1.2</td>
<td>3.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gnb5</td>
<td>1.2</td>
<td>2.6</td>
<td>1.2</td>
<td>3.3</td>
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<tr>
<td>Gca1a</td>
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<td>9.6</td>
<td>8.5</td>
<td>7.1</td>
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<tr>
<td>Galk1</td>
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<td>1.3</td>
<td>3.2</td>
<td>4.5</td>
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<td></td>
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<td>Mil</td>
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<td>1.7</td>
<td>1</td>
<td>1.1</td>
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<tr>
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<td>0.5</td>
<td>0.4</td>
<td>0.5</td>
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<tr>
<td>Omp1mow</td>
<td>3</td>
<td>4.7</td>
<td>4.2</td>
<td>3.9</td>
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<tr>
<td>Pde2</td>
<td>1.2</td>
<td>1.6</td>
<td>1.2</td>
<td>0.9</td>
<td></td>
<td></td>
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<tr>
<td>Pde6b</td>
<td>1.6</td>
<td>1.5</td>
<td>2.7</td>
<td>1.5</td>
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<td></td>
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<tr>
<td>Pde6g</td>
<td>2.2</td>
<td>1.8</td>
<td>2.9</td>
<td>1.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prom1</td>
<td>1.3</td>
<td>1.1</td>
<td>0.8</td>
<td>0.7</td>
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<td></td>
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<tr>
<td>Rho</td>
<td>3.1</td>
<td>3.5</td>
<td>7.3</td>
<td>2.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rom1</td>
<td>1.7</td>
<td>1.3</td>
<td>0.6</td>
<td>6.1</td>
<td></td>
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</tr>
<tr>
<td>Sag</td>
<td>1.9</td>
<td>1.4</td>
<td>4.1</td>
<td>0.6</td>
<td></td>
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</tr>
<tr>
<td>Sgp</td>
<td>1.4</td>
<td>8.1</td>
<td>1.1</td>
<td>9.6</td>
<td></td>
<td></td>
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<tr>
<td>Unc119h</td>
<td>1.5</td>
<td>1.5</td>
<td>0.5</td>
<td>8.4</td>
<td></td>
<td></td>
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<tr>
<td>Uspl2</td>
<td>1.2</td>
<td>4.7</td>
<td>3.2</td>
<td>2.2</td>
<td></td>
<td></td>
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<tr>
<td>Vtn</td>
<td>1.9</td>
<td>11.6</td>
<td>0.0</td>
<td>43.8</td>
<td>0.0</td>
<td></td>
</tr>
</tbody>
</table>

Verification by real-time PCR and in situ hybridization. To independently validate our Affymetrix data, we compared results of real-time PCR analyses with those of Affymetrix analyses. We randomly chose to analyze 18 genes from analysis II and III whose levels of expression shown by our Affymetrix analyses vary widely from 0.2- to 19.2-fold between Rpl1<sup>+/+</sup> and Rpl1<sup>-/-</sup> mice. We used three independent RNA samples derived from six to eight retinas or from three to four different mice for each time point and genotype for analysis by real-time PCR under identical conditions. We normalized the real-time PCR results to those of the 18S rRNA gene and calculated the ratio of Rpl1<sup>+/+</sup> to Rpl1<sup>-/-</sup> values (Supplementary Material, Table S5). Among 22 comparisons in 18 genes between PCR-derived and Affymetrix-derived ratios, 15 (68.2%) showed the same trends (i.e. higher or lower expression levels in Rpl1<sup>-/-</sup> than in Rpl1<sup>+/+</sup> retinas as indicated by ratios that were >1 or <1, respectively). For genes that displayed >1.3-fold increases in Affymetrix analysis, all 12 (100%) have been confirmed to have the same trends using real-time PCR. We performed Student’s t-test on the real-time PCR results. Among the 15 of 18 genes that showed the same trends with Affymetrix result, we found seven were significantly changed (P < 0.05, Student’s t-test), as indicated by asterisks in Supplemental Material, Table S5. The real-time PCR did not show much significance (seven out of 18) may be due to the variation among three
independent replicates for each gene at different dates and using reagents from different companies plus biological variations.

To provide proof that the two independent methods gave similar results, we created a scatter plot of the average fold changes from real-time PCR versus fold changes from Affymetrix. The correlation coefficient of the two variables was 0.985 (P < 0.001). We also performed the regression of mean fold changes of Affymetrix results on the mean fold changes of real-time PCR results, the regression coefficient was 1.284 (P < 0.001) and R^2 was 0.97. To exclude the potential influence of the putative outlier, we re-performed the same analyses by excluding the single data point of Edn2 and found that the correlation coefficient of the two variables was 0.85 (P < 0.0001), the regression coefficient was 1.14 (P < 0.0001) and R^2 was 0.72 (Fig. 2). Therefore, these two variables are highly correlated. The results of such analyses are consistent with our results (Table 2 and Fig. 1) comparing to SAGE results and results of Dorrell et al. and consistent with those in other reports (25,26).

To verify that the genes identified by Affymetrix analyses are indeed expressed in the retina and photoreceptors, and to determine whether any of the genes we identified had altered expression patterns or levels in mutant retinas and photoreceptors, we examined the expression of 23 genes by using in situ hybridization. These genes were chosen from analysis III (majority of which were also identified in analysis II) and overlap with genes selected for real-time PCR analysis, but their Affymetrix signal intensities again vary in wide ranges. We used anti-sense riboprobes labeled with the isotope P^33 for hybridization so that intensity could be quantified for direct comparison between Rp1^+/+ and Rp1^+/-. Because of the clear layer structure of retinas in Rp1^+/+ and Rp1^-/- mice at these ages, we could determine approximately the subcellular localization of the riboprobes in photoreceptors in outer nuclear layer and other layers. All 23 genes that we analyzed did have expression signals in retinas and 20 were expressed in photoreceptors at detectable levels, whereas the other three were expressed at very low levels close to the background (Supplementary Material, Fig. S1). We used the Image-Pro Plus to quantify the densities of in situ signals across the entire retinas from OS to ganglia relative to backgrounds and analyzed the fold changes between Rp1^+/+ and Rp1^-/- retinas at corresponding ages. We identified 15 of 23 (65.2%) genes that showed consistent trends of fold changes with those of Affymetrix analysis. Among them, six genes displayed changes between Rp1^-/- and Rp1^+/+ in both photoreceptor cells and non-photoreceptor cells in same directions as in whole retinas (data not shown). Owing to the intrinsic limitations of semi-quantitative in situ hybridization, the fold changes we obtained here cannot be considered as quantitative proof of the Affymetrix results. However, taken together, the results of our statistical analyses and verifications by using real-time PCR demonstrate that our Affymetrix expression profiles of Rp1^+/+ and Rp1^-/- retinas between P7 and P21 are fairly accurate.

Distinct, multiple molecular phenotypes in Rp1^-/- retinas at different time points

From our analysis II that identified genes with significant changes between Rp1^+/+ and Rp1^-/- at each of five time points (Table 1), we further classified these genes according to the functional characteristics of the gene products by using the Affymetrix gene ontology tools and determined the proportions of genes (up- or down-regulated in Rp1^-/-) in each functional category (Fig. 3). To determine whether changes in gene ontology analyses in differentially expressed genes we identified are significant
compared with those of the entire Affymetrix array, we performed an Expression Analysis Systematic Explorer analysis (27) and found that the functional category changes in differentially expressed genes we identified were not statistically significant. This finding is not contradictory to the real-time PCR and microarrays results. Even if one gene in each functional category was significantly differentially expressed, no significant changes were necessary for each functional category. This analysis clearly showed that the molecular defects in \( \text{Rp}1^{-/-} \) would be necessary for each functional category. This analysis showed that the molecular defects in \( \text{Rp}1^{-/-} \) retinas involve multiple molecular pathways, as reported previously in the analyses of \( \text{Crx}^{-/-} \), \( \text{Nrl}^{-/-} \) and \( \text{Pde6brd1}^{+/+} \) retinas (24,26,28).

Surprisingly, as early as P7, \( \text{Rp}1^{-/-} \) retinas have already undergone significant molecular changes in multiple biological pathways relative to \( \text{Rp}1^{+/+} \) retinas. In addition, we found that very few genes that were differentially expressed in \( \text{Rp}1^{+/+} \) and \( \text{Rp}1^{-/-} \) retinas were differentially expressed at all five developmental stages in our analysis II. This finding suggests that the molecular responses to the disruption of \( \text{Rp}1 \) change markedly between P7 and P21. Functional categorization of these genes revealed the temporal pattern of disease progression (Fig. 3). Excluding the ‘unknown’ category, the largest numbers of significantly changed genes moved from ‘metabolism’ at P7, ‘transcription’ or ‘transport’ at P10, ‘response to stimulus’ at P14 and ‘metabolism’ at P18 to ‘signal transduction’, ‘transcription’ or ‘transport’ at P21.

\( \text{Rp}1^{-/-} \) retinas display molecular responses that are distinct from those of \( \text{Crx}^{-/-} \), \( \text{Nrl}^{-/-} \) and \( \text{Pde6brd1}^{+/+} \)

Among many mouse models of RP, \( \text{Crx}^{-/-} \), \( \text{Nrl}^{-/-} \) and \( \text{Pde6brd1}^{+/+} \) share several common features with \( \text{Rp}1^{-/-} \) : OS abnormality and progressive degeneration of photoreceptors (26,29,30). Moreover, gene expression profiles of \( \text{Crx}^{-/-} \), \( \text{Nrl}^{-/-} \) and \( \text{Pde6brd1}^{+/+} \) are available at P10 or P14 for comparison (24,26,28). Therefore, we compared our Affymetrix results at P10 with SAGE results from \( \text{Crx}^{-/-} \) mice at P10.5 and Affymetrix results from \( \text{Nrl}^{-/-} \) mice at P10. The \( \text{Rp}1^{-/-} \), \( \text{Crx}^{-/-} \) and \( \text{Nrl}^{-/-} \) mutants had almost completely different sets of genes that were differentially expressed between \( \text{Rp}1^{-/-} \) and \( \text{Rp}1^{+/+} \) retinas. Of the genes shown by SAGE to be differentially expressed in \( \text{Crx}^{-/-} \) and \( \text{Crx}^{+/+} \) retinas at P10.5, and 150 genes are identifiable by U74Av2 arrays. Only 10 (<7%) of these 150 genes were identified in our \( \text{Rp}1 \) analysis III at P10 (Supplementary Material, Table S6). Furthermore, SAGE- and Affymetrix-derived ratios indicating increased or decreased expression of these 10 genes in \( \text{Crx}^{-/-} \) and \( \text{Crx}^{+/+} \) retinas at P10.5, and 150 genes are identifiable by U74Av2 arrays. Only 10 (<7%) of these 150 genes were identified in our \( \text{Rp}1 \) analysis III at P10 (Supplementary Material, Table S6). Similarly, among 173 genes that were significantly changed at P10 between \( \text{Nrl}^{+/+} \) and \( \text{Nrl}^{-/-} \) (28), none (except 1110008H02Rik) overlapped with those that were significantly changed between \( \text{Rp}1^{+/+} \) and \( \text{Rp}1^{-/-} \) at the corresponding age.

We also compared our results of \( \text{Rp}1^{-/-} \) to those of \( \text{Pde6brd1}^{ad} \) at a comparable age (P14). Among 167 genes that are significantly changed in \( \text{Pde6brd1}^{ad} \) at P14 (26), 48 have corresponding probe sets on U74Av2 microarrays and only one gene (guanine nucleotide-binding protein, \( \beta1 \) subunit) is common between \( \text{Rp}1^{-/-} \) and \( \text{Pde6brd1}^{ad} \). This gene is down-regulated in both disease models. Notice that \( \text{Rp}1 \) is significantly down-regulated in \( \text{Pde6brd1}^{ad} \) at P14 (26).
Specific pathways in Rp1<sup>−/−</sup> retinas

In our analyses III, we identified 340 genes whose expression levels are significantly affected by the interaction between development and genotype at the early phase of disease progression. Therefore, it is likely that these 340 genes are directly involved in pathways specific for Rp1 normal function. We used a pathway-finding program (Ingenuity Pathways, www.analysis.ingenuity.com) to analyze the functions of these genes. The Ingenuity Pathways is one of the largest curated databases of biological networks created from millions of individually modeled relationships between proteins, genes, complexes, cells, tissues, drugs and diseases. We retrieved 157 genes from these 340 genes that had records in the Ingenuity Pathways database.
Among multiple pathways identified in our Ingenuity analysis, we surprisingly found genes involved in JNK signaling cascades (Table 3). The JNK pathway consists of the core members of kinases and their peripheral substrates and interacting partners; among 340 genes we identified, two (Map3k7 and Map4k3) are core members of the kinases and 12 are peripheral substrates and interacting partners (Table 3) (31–38). To confirm that these genes were indeed differentially expressed, we performed real-time PCR on seven of them at various time points and eight of 11 (73%) comparisons showed consistent changes between real-time PCR and Affymetrix results (Table 3), whereas seven comparisons were significantly different (Student’s t-test, P < 0.05), which is consistent with our previous verification on randomly chosen genes by real-time PCR (Fig. 2 and Supplementary Material, Table S5).

**Confirmation of compromised JNK signaling in Rp1<sup>−/−</sup> retinas**

Because many genes found in our microarrays analysis are peripheral targets of JNK signaling cascades, we reasoned that altered JNK signaling cascades could explain the changes of these JNK target genes. Although many core members of JNK cascades did not display significant changes in their mRNA levels, we investigated active JNKs and other key members of JNK signaling cascades in retinas at P21. As shown in Figure 4, the phosphorylation of both JNK1 and JNK2 is significantly decreased at P21 in the Rp1<sup>−/−</sup> retinas, whereas the total amounts of JNK1 and JNK2 in Rp1<sup>−/−</sup> are similar to those in Rp1<sup>+/+</sup> mice. The phosphorylation of c-Jun is slightly reduced in Rp1<sup>−/−</sup> at P18–21, whereas that of MKK4 is also decreased in Rp1<sup>−/−</sup> retinas at P18–21; however, neither of them is statistically significant (data not shown).

The axoneme in photoreceptors in Rp1<sup>−/−</sup> retinas is much shorter than that in Rp1<sup>+/+</sup> animals (11), suggesting that the assembly of microtubules in photoreceptors in Rp1<sup>−/−</sup> is compromised. MAP2 is known to be a substrate of JNK and involved in regulating microtubule dynamics of the axoneme in photoreceptors (39). Therefore, we examined the phosphorylation of MAP2 in Rp1<sup>−/−</sup> retinas using Western blot analysis. Because the serine residue 136 of MAP2 is very likely the substrate of JNK (40,41), we chose to use the phospho-serine 136-MAP2 antibody. Figure 4 shows that the phosphorylation of MAP2 is indeed significantly reduced in Rp1<sup>−/−</sup> retinas at P21.

Furthermore, to address whether other MAP kinase pathways (Erk and p38) related to the JNK pathway are also reduced in Rp1<sup>−/−</sup> retinas, we performed similar Western analyses with phospho- and pan-ERK (Fig. 4C and D) and p38 antibodies (data not shown) at P18 and P21 and found no significant changes.

To provide evidence that the key members of the JNK signaling cascades examined earlier are indeed active in photoreceptors, we performed immunofluorescence using phospho-specific antibodies on retinas of Rp1<sup>+/+</sup> and Rp1<sup>−/−</sup> at P18 and P21. Although no significant differences in fluorescent intensities were detected between Rp1<sup>+/+</sup> and Rp1<sup>−/−</sup> (likely due to the limitation of immunofluorescence detection), phosphorylated JNK1, JNK2 and MAP2 are present in photoreceptors at these ages (Fig. 5) (P18 data not shown). Specifically, phospho-JNK1, -JNK2 and -MAP2 immunofluorescence signals were detected mostly in the OS.

**DISCUSSION**

**Molecular mechanisms in Rp1<sup>−/−</sup>**

**Molecular mechanisms in Rp1<sup>−/−</sup> are distinct from those in Crx<sup>−/−</sup>, Nrl<sup>−/−</sup> and Pde6b<sup>−/−</sup>**

The phenotypes of retinal degenerative diseases share a common endpoint: photoreceptor cell death, while their...
Figure 4. Phosphorylation of JNK1, JNK2 and MAP2 is decreased in Rp1\(^{-/-}\) retinas, whereas phosphorylation of ERK has no changes. Western blots of retina extracts from Rp1\(^{+/+}\) and Rp1\(^{-/-}\) at P18 and P21 were probed sequentially with phospho-JNK1/2, pan-JNK1, JNK2-D2, phospho-MAP2 and MAP2 antibodies (A), phospho- and pan-ERK antibodies (C), with GAPDH as control. Relative values of phosphorylated JNK1, JNK2, MAP2 (B) and ERK (D) in Rp1\(^{+/+}\) and Rp1\(^{-/-}\) retinas at P18 and 21 are plotted in the way that intensity ratios of phospho-protein to corresponding pan-protein were calculated relative to the average ratio of Rp1\(^{+/+}\) mice as '1'. Three independent sets of mice were analyzed. Relative to GAPDH, pan-proteins are not significantly different between Rp1\(^{+/+}\) and Rp1\(^{-/-}\) at all time points (data not shown). Asterisk indicates statistical significance (Student’s t-test, \(P < 0.05\)).
The results of immunostaining with phospho-MAP2 antibody. Phospho-MAP2 is present in the photoreceptor OS of the both Rp1 when compared with the negative control (A and D), for which competitive peptide was used to bind the antibody before the primary antibody incubation. (G – L) The results of immunostaining with phospho-MAP2 antibody. Phospho-MAP2 is present in the photoreceptor OS of the both Rp1+/- (H and K) and Rp1-/- retinas (I and L). In the negative control (G and J), we used the secondary antibody only. To show the immunostaining clearly, the same sections are shown with (A–C, G–I) and without DAPI staining (D–F, J–L). Red: DAPI staining for nuclei; Green: phospho-proteins. ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. Bar = 40 μm.

Figure 5. Immunofluorescence of phospho-JNK1/2 and phospho-MAP2 in Rp1+/- and Rp1-/- photoreceptors at P21. (A–F) The results of immunostaining with phospho-JNKs antibody. It is clear that phospho-JNKs are present in the photoreceptor OS of the both Prph2Rds (46), compared our Rp1 retinal diseases that have been published recently, here we further compare molecular profiles of other mouse mutant strains with photoreceptor OS degeneration and/or rhodopsin mislocalization: rhodopsin-/- (43,44), rd7 (45), Rom1-/- (46), Prph2Rds+/- or Rds-/- (47), Nna1Pcmd (48), Rpgr-/- (49), Myo7a-/- (50) and Tulp1-/- (51). Particularly, it would be interesting to examine JNK signaling in these mutants as well as in Crx-/-, Nrl-/- and Pde6b-4d. Consistent with our findings, recent studies of Huntington's and Parkinson's disease models in yeast have also indicated that despite similarities between the two degenerative diseases at cellular levels, non-overlapping sets of conserved genes and pathways are involved in the regulation of the disease phenotypes (52). Moreover, analogous to molecular profiling in cancer diagnosis and treatment (53), the molecular profiles of Rp1-/- disease and other mouse models of RP could help define prognosis and identify therapeutic interventions for these diseases.

Defects in JNK signaling cascades in Rp1-/- retinas

We focused our analysis on five time points when Rp1 disease is still in its early phase, and on changes that are statistically significant either at each time point between two genotypes (analysis II) or in the interactions between five time points and two genotypes (analysis III). In analysis II, there is surprisingly little overlap (~2%), between two adjacent time points, of genes that are expressed at significantly different levels in Rp1-/- and Rp1+/+ retinas. Furthermore, different genes are common to different pairs of time points. These results suggest that molecular responses induced by the Rp1 mutation change dramatically between P7 and P21. From our analyses of normal retinas (analysis I), it is clear that during the second and third postnatal weeks, photoreceptors undergo rapid structural and molecular changes. These changes could explain the surprisingly small overlap among the five time points in Rp1-/- retinas.

Our analysis III identified 340 genes whose expression differed significantly depending on genotype and developmental age. Therefore, these genes are highly likely to have a role in the normal and pathologic pathways involving Rp1. By using pathway and subsequent experimental analyses of these 340 genes, we provided several lines of compelling evidence that the JNK signaling cascades are severely affected in Rp1 diseases and thus directly related to the normal function of Rp1.
First, analysis III identified a number of peripheral targets (Table 3) of JNK signaling cascades that displayed significant changes in their mRNA levels in Rp1−/− and Rp1+/+ retinas. Furthermore, these changes are correlated between genotype and time, thus indicating the defective common pathways of JNK signaling. We further confirmed these changes by using real-time PCR analyses of independent retina samples.

Secondly, we confirmed that the phosphorylation of JNK1 and JNK2 was significantly reduced at P21 in Rp1−/− retinas when compared with that in Rp1+/+ retinas, whereas pan-JNK1/JNK2 remained unchanged. Furthermore, phospho-MAP2 (a substrate of JNK signaling) is also reduced significantly at P21 in Rp1−/− retinas. The reductions of phospho-c-Jun and phospho-MKK4 at P18–21, although not statistically significant, are correlated and further corroborate with the overall reduction of JNK signaling cascades in Rp1−/− retinas. Moreover, the RI1 diseases reduce JNK signaling specifically, because the other two MAP kinase pathways (Erk and p38) related to the JNK signaling cascades have no significant changes. These results strongly demonstrated that the RI1 diseases reduce JNK signaling specifically and warranted further mechanistic investigations.

It is noted that many genes in Table 3 showed increased expression levels in Rp1−/− retinas at P21. How these changes are related to the decreased phosphorylated JNKs remain to be investigated. JNK signaling cascades are known to regulate the activities of many transcription factors in a complex manner. For example, JNKs can phosphorylate c-Jun and other AP-1 components, therefore activate the transcriptional activities, whereas JNKs are also known to inhibit the transcriptional activities of NFAT4 (54,55). A recent report stated that JNK1 and JNK2 regulate the expression of P53 in opposite manners: JNK1 decreases (54,55). A recent report stated that JNK1 and JNK2 regulate the expression of P53 in opposite manners: JNK1 decreases whereas JNKs are also known to inhibit the transcriptional activities of many transcription factors in a complex manner. For example, JNKs can phosphorylate c-Jun and other AP-1 components, therefore activate the transcriptional activities, whereas JNKs are also known to inhibit the transcriptional activities of NFAT4 (54,55). A recent report stated that JNK1 and JNK2 regulate the expression of P53 in opposite manners: JNK1 decreases whereas JNKs are also known to inhibit the transcriptional activities of many transcription factors in a complex manner. For example, JNKs can phosphorylate c-Jun and other AP-1 components, therefore activate the transcriptional activities, whereas JNKs are also known to inhibit the transcriptional activities of NFAT4 (54,55).

A model of RI1 function in development and diseases

Rp1 activates JNK cascades through rhodopsin and Rac1 (Fig. 6). Given the OS disorganization, mislocalization of rhodopsin and progressive cell death in Rp1−/− photoreceptors and the localization of Rp1 in the OS axoneme as a MAP, a link between RI1 and JNK cascades may be established. The disruption of RI1 in photoreceptors could lead to the mislocalization of rhodopsin, which then interferes with the activation of Rac1 upon the light stimulus, ultimately reducing the activities of the JNK signaling cascades including MKK4, JNK1 and JNK2, disturbing the actin cytoskeleton and finally, culminating in neurodegeneration. In vertebrate photoreceptors, rhodopsin can activate Rac1, a member of the small GTPase family (58) that is a known activator of JNKs and plays important roles in the regulation of the actin cytoskeleton, gene expression, and protein and vesicular transport (58–61). Alternatively, it is possible that activities of JNK-related phosphatases could be increased in Rp1−/− photoreceptors, leading to the reduction of phosphorylated JNKs (62).

Interestingly, kinesins (microtubule-associated motors) also bind to molecules in the JNK pathway (such as JNK interacting protein 1 or JIP1) for vesicle trafficking (12); kinesin II is involved in transport of rhodopsin in photoreceptors (63). Similar to kinesins, it is, therefore, possible that RI1 directly interacts with microtubules and members of JNK cascades. Like DCX and its regulations through JNK pathways in neurons (12,64–66), RI1 contains at least three evolutionarily conserved consensus sites for phosphorylation by JNK with nearby characteristic K, R or H residues (67), suggesting that RI1 could be a direct target of JNK signaling cascades. Therefore, there could be a two-way talk between RI1 and JNK cascades: RI1 might be phosphorylated and thereby regulated by JNK signaling cascades and RI1 could have a feedback regulation on the JNK cascades. However, how JNK regulates RI1 and how Rp1−/− alters the JNK targets at the transcriptional level remain to be investigated. In support, the oxygen level regulates both the RI1 transcription (which led to the original identification of the RI1 gene) and the JNK pathway (3,68).

Hypo-phosphorylation of JNK targets (MAP2 and c-Jun) in Rp1−/− retinas. In vitro, MAP2 can be phosphorylated by JNK1 and in Jnk1−/− mouse brain, the phosphorylation of MAP2 is decreased (41). Consistent with the decrease in the phosphorylation of JNKs, MAP2 in Rp1−/− retinas is also hypo-phosphorylated, contributing to the shorter length of axoneme in Rp1−/− than that in Rp1+/+ retinas. Therefore, RI1 and MAP2 have similar but non-overlapping function in photoreceptor microtubule dynamics and both can be regulated by JNK signaling cascades. It remains to be determined whether the phosphorylation of RI1 is similarly reduced in Jnk1−/− photoreceptors.

In mammals, JNKs display both pro- and anti-apoptotic effects. For example, Jnk1−/− Jnk2−/− double knockout
mice die as embryos, which have been attributed to an increase in apoptosis of the forebrain and decrease in apoptosis in the hindbrain (69,70). Jnk3−/− mice show increased resistance to kainic acid-induced seizures and thus reduced cell death of hippocampal neurons (71). Similar decreases in cell death are found in mice expressing a mutant form of c-Jun, whose phosphorylation sites by JNK are replaced with alanines (72), strongly suggesting that JNK signaling cascades regulate cell death through the downstream transcription factors, such as c-Jun. Thus, the reduction of JNK signaling cascades in Rp1−/− could trigger the subsequent photoreceptor cell death through reduction of the c-Jun activity, although currently we do not know whether phospho-c-Jun remains reduced at later stages (after P21). In addition to c-Jun, members of the Bcl-2 family, such as Bcl-2 and Bcl-XL, could play either apoptotic or anti-apoptotic roles when phosphorylated by JNK (73,74).

Therapeutic strategies for RP1 diseases
Our identification of decreased JNK signaling cascades in Rp1−/− photoreceptors also suggests that JNK-specific activators rather than inhibitors may be efficient therapeutic agents for RP1 diseases. A number of JNK-specific inhibitors and activators available commercially could be tested at specific times in Rp1−/− mice for their effects on retinal phenotypes. Moreover, analogous to overexpression of Rac1 in rescuing retinal phenotypes in Drosophila with mutant rhodopsin, activation of the JNK signaling cascades by over-expressing upstream activators such as Rac1 may represent an alternative strategy for partially reducing the progression and delaying the onset of the RP1 diseases (75).

MATERIALS AND METHODS
Preparation of mouse retinas
All animals were handled in accordance with the NIH Guide for Care and Use of Laboratory Animals, St Jude Children’s Research Hospital’s policies and with the Association for Research in Vision and Ophthalmology statement for the use of animals in ophthalmic and vision research. We used Rp1+/+ and Rp1−/− mice maintained in brother–sister breeding and of mixed C57Bl/6 and 129SvEv background in generations F3–F5. The sex of the animals was not determined. Rp1−/− and Rp1+/+ mice were genotyped as described previously (9). Neural retinas were collected at the same time of day (1–2 p.m.) from P7, P10, P14, P18 and P21 mice, and for each hybridization, RNA was isolated from neural retinas pooled from three or four mice. Hybridizations were performed in triplicate for each age and genotype (a total of 30 hybridizations). For in situ hybridization, western blot and immunostaining, retinas or eyes of individual mice were collected at the same time of day (1–2 p.m.) and processed in identical conditions; only littermates were used for comparison.

Preparation and labeling of RNA
Total RNA was purified by using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. The amount and quality of each RNA preparation were determined by UV spectrophotometry. The absorbance readings of all samples at 260 and 280 nm gave ratios between 1.8 and 2.1. The integrity of the RNA was determined using an Agilent 2100 Bioanalyzer where the ratio of 28S to 18S peaks was approximately two for each sample. cDNA was first synthesized and then cRNA was synthesized and labeled according to the manufacturer’s protocols (Affymetrix, Santa Clara, CA, USA).

Microarray hybridization
We used Affymetrix U74Av2 microarrays containing 12,488 probe sets corresponding to ~6,000 known genes and 6,000 EST clusters for each hybridization. The labeled RNA was fragmented and hybridized to the chips according to Affymetrix protocols. Microarrays were scanned and the expression value for each probe set was calculated by using software supplied by Affymetrix (Microarray Suite 5.0).

Data analysis
Before performing statistical comparisons, we rescaled the signals obtained by hybridization with each probe set so that the median signal from all probe sets on each array was 850 and natural logarithm (base e) was applied to the signals. Three different analysis methods were used: Student’s t-test, one-way ANOVA and two-way ANOVA. The optimal significance (α) levels and the corresponding FDR, were determined by the profile information criterion and the coupled FDR estimator (23).

Figure 6. Model of Rp1 and JNK in photoreceptors. For details, refer Discussion section.
Real-time PCR

Real-time PCR was performed with gene-specific primers and probes according to the method described previously (76); primers and probes are listed in Supplementary Material, Table S7. RNA samples used in real-time PCR analysis were different from those used in Affymetrix analyses. All real-time PCR were performed in triplicate.

In situ hybridization

Eyes from Rp1\(^{+/+}\) and Rp1\(^{-/-}\) mice were embedded in tissue freezing medium (Triangle Biomedical Sciences, Durham, NC, USA) and cut into 18 \(\mu\)m sections in a cryostat at \(-23^\circ\)C. Genes that were used for in situ hybridization are in Supplementary Material, Table S7 and the protocol was described previously (48). Image-Pro Plus software (Media Cybernetics, San Diego, CA, USA) was used to quantify the densities of in situ signals across the entire retinas from OS to ganglia relative to backgrounds, and the fold changes between Rp1\(^{+/+}\) and \(-/-\) retinas at corresponding ages were analyzed. To ensure the pictures of both the Rp1\(^{+/+}\) and Rp1\(^{-/-}\) sections side-by-side in the same embedding blocks. The pictures for each gene of both Rp1\(^{+/+}\) and Rp1\(^{-/-}\) were taken under the same conditions.

Protein extraction and Western blot

Rp1\(^{+/+}\) and Rp1\(^{-/-}\) mouse retinas were homogenized in buffer containing 50 mM Tris–Cl (pH 8.0), 150 mM NaCl, 15 mM EDTA and 1x protease inhibitor cocktail (Roche, Indianapolis, IN, USA). The homogenate was centrifuged at 12 000g for 15 min at 4°C, and the supernatant was collected and boiled in SDS sample buffer, then separated by SDS–PAGE (12%). Proteins were then transferred to polyvinylidene difluoride membrane. The membrane was blocked in 5% non-fat milk (Bio-Rad, Hercules, CA, USA) at room temperature for 1 h and incubated with phospho-specific JNK1 and JNK2 (pTpY183/185) antibody (1:1000, Biosource, Camarillo, CA, USA), JNK1 pan antibody (1:600, Biosource), JNK2 D2 antibody (1:1000, Santa Cruz, Santa Cruz, CA, USA), phospho-MKK4 (Ser257/Thr261) antibody (1:1000, Cell Signaling Technology, Beverly, MA, USA), phospho-MAP2 (Ser136) antibody (1:1000, Cell Signalling Technology), MAP2 antibody (1:5000, Cell Signaling Technology), phospho-c-Jun (Ser73) antibody (1:1000, Cell Signalling Technology), phospho-ERK antibody, phospho-p38 antibody (1:500, Cell Signaling Technology), ERK1 (C-20) antibody (1:500, Santa Cruz) or GAPDH antibody (1:5000, Abcam, Cambridge, MA, USA) at room temperature for 1 h or overnight at 4°C. The signal was detected by incubating with secondary antibody, HPR-conjugated anti-rabbit, anti-mouse (1:6000, Amersham Biosciences, Piscataway, NJ, USA) or anti-chicken (1:1000, Santa Cruz) and visualized using ECL or ECL advances (Amersham Biosciences). For quantification, linear ranges of exposure times of each antibody were determined experimentally and at least two different exposure times within the linear ranges were taken for each antibody. Intensities of each band measured by using the software Fluorchem 8900 (Alpha Innotech, San Leandro, CA, USA) were subtracted from those of backgrounds and normalized to those of GAPDH. Percentages in Rp1\(^{-/-}\) were normalized to those in Rp1\(^{+/+}\) controls.

Immunofluorescence

Eyes of Rp1\(^{+/+}\) and Rp1\(^{-/-}\) littermates were enucleated and fixed in 4% paraformaldehyde solution in 0.1 M phosphate buffer (pH 7.4), embedded in OCT with both genotypes side-by-side in same blocks and cryosectioned at 12 \(\mu\)m. Slides were stained with primary antibodies (1:100 for phospho-JNK antibody and phospho-MAP2 antibody). For the competitive experiment of phospho-JNK1 and -JNK2 immunostaining, various concentrations of the peptide antigen (Cell Signaling Technology) were used (2, 4 and 10 \(\mu\)l). Reactions were visualized by the treatment of FITC-conjugated secondary antibody and then observed under fluorescence microscopy.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

DATA DEPOSITION


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Conflict of Interest statement. None declared.

REFERENCES


