RASGRF2 controls nuclear migration in postnatal retinal cone photoreceptors

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ABSTRACT
Detailed immunocytochemical analyses comparing wild-type (WT), GRF1-knockout (KO), GRF2-KO and GRF1/2 double-knockout (DKO) mouse retinas uncovered the specific accumulation of misplaced, ‘ectopic’ cone photoreceptor nuclei in the photoreceptor segment (PS) area of retinas from GRF2-KO and GRF1/2-DKO, but not of WT or GRF1-KO mice. Localization of ectopic nuclei in the PS area of GRF2-depleted retinas occurred postnatally and peaked between postnatal day (P)11 and P15. Mechanistically, the generation of this phenotype involved disruption of the outer limiting membrane and intrusion into the PS layer by cone nuclei displaying significant perinuclear accumulation of signaling molecules known to participate in nuclear migration and cytoskeletal reorganization, such as PAR3, PAR6 and activated, phosphorylated forms of PAK, MLC2 and VASP. Electrotetroretinographic recordings showed specific impairment of cone-mediated retinal function in GRF2-KO and GRF1/2-DKO retinas compared with WT controls. These data identify defective cone nuclear migration as a novel phenotype in mouse retinas lacking GRF2 and support a crucial role of GRF2 in control of the nuclear migration processes required for proper postnatal development and function of retinal cone photoreceptors.

KEY WORDS: Ras signaling, GEF, GRF1, GRF2, Retina, Cone photoreceptor, Nuclear migration, Intracellular traffic

INTRODUCTION
The RASGRF family of mammalian guanine nucleotide exchange factors (GEFs) has two highly homologous members, GRF1 and GRF2 (also known as RASGRF1 and RASGRF2), that are preferentially expressed in the central nervous system (CNS) and are able to activate different members of the Ras or Rho/Rac subfamilies of small GTPases in a variety of cellular contexts. Because of their high structural homology and similar expression patterns, the specific functional properties of the two isoforms within this mammalian GEF family are not fully defined (Feig, 2011; Fernández-Medarde and Santos, 2011). Previous characterization of GRF1-knockout (KO) mice has documented a specific implication of this protein in memory formation (Brambilla et al., 1997; Giese et al., 2001), postnatal growth (Itier et al., 1998; Fernández-Medarde et al., 2002), pancreatic β-cell function and glucose homeostasis (Font de Mora et al., 2003; Manyes et al., 2014), and neurosensory processes and photoreception (Fernández-Medarde et al., 2007, 2009; Hysi et al., 2010). By contrast, the study of Rasgrf2-null animals has demonstrated that GRF2 and GRF1 are not functionally overlapping regarding memory formation or body growth (Fernández-Medarde et al., 2002; Jin et al., 2013; Li et al., 2006) and has also documented that GRF2 plays specific functional roles in control of T-cell proliferation and signaling in lymphocyte proliferation, T-cell signaling responses and lymphomagenesis (Ruiz et al., 2007, 2009). Recent studies of specifically affected human patient subpopulations support a role of GRF1 in predisposition to myopia and refractive errors of vision (Hysi et al., 2010) and of GRF2 in predisposition to addictive alcohol abuse (Easton et al., 2014; Schumann et al., 2011; Stacey et al., 2012).

In view of our previous observations in GRF1-KO mice, supporting the relevance of GRF1 in mammalian visual processes (Fernández-Medarde et al., 2009; Hysi et al., 2010), we decided to carry out more detailed, in-depth analyses of visual phenotypes as well as eye structure and development in the GRF1-KO or GRF2-KO mouse strains generated in our laboratory. In order to clearly discriminate between possible specific functionalities of each of these two members of the GRF family, our experimental approach involved parallel studies of comparable experimental sample sets originating from animals sharing the same genetic background and corresponding to four distinct, relevant GRF genotypes, including wild type (WT), Rasgrf1−/− (GRF1-KO), Rasgrf2−/− (GRF2-KO) and Rasgrf1−/− Rasgrf2−/− (GRF1/2-DKO) animals.

Here, we describe histological alterations occurring specifically in the retinas of GRF2-KO and GRF1/2-KO mice that were absent in those of the other relevant GRF genotypes analyzed. In particular, the observed histological alterations affect specifically the subpopulation of retinal cone photoreceptors and are indicative of the occurrence of significant alterations of the process of nuclear migration in this particular photoreceptor cell type when GRF2 is absent. In the vertebrate retina, the so-called late migratory phase is a still poorly understood process from the mechanistic point of view that is essential for correct photoreceptor differentiation and establishment of functional synaptic connections during development of the retina in mammals (Rich et al., 1997). This report describes recent studies from our laboratory probing the eyes of GRF-knockout mice by means of electrotetroretinogram (ERG) assays and immunohistochemical analyses using markers for different cellular signaling molecules that document a crucial contribution of GRF2 to the nuclear migration processes needed for correct postnatal development and function of cone photoreceptors in the mammalian retina.

RESULTS
Specific detection of ectopic nuclei in the photoreceptor segment area of retinas of GRF2-depleted mice
To detect whether the retinas of animals devoid of one or both isoforms of the GRF family of exchange factors display any specific
structural alterations, we performed in-depth immunohistochemical analyses of retinas obtained from adult mice of four relevant genotypes (WT, GRF1-KO, GRF2-KO, GRF1/2-DKO) that shared the same genetic background and were raised and bred in parallel in our mouse colony for these experiments.

Strikingly, the only consistent alteration observed after our initial, extensive analysis of retinas from 3- to 5-month-old adult animals was the specific presence, in retinas of GRF2-depleted mice, of displaced, ectopic nuclei located in the photoreceptor segment (PS) area of the retinas (Fig. 1A-D, Hoechst 33342, arrows). Thus, whereas the PS area is completely devoid of nuclei in the WT controls, and also in the GRF1-KO retinas (Fig. 1A,B), a sizable, significant number of abnormally located, ectopic nuclei (about 50–60 nuclei per individual 16–18 µm tissue section) was consistently detected in that particular layer of the GRF2-KO and GRF1/2-DKO adult retinas examined (Fig. 1C,D). However, the histological structure of all other retinal cell layers appeared indistinguishable between WT mice and all GRF-Mouse genotypes analyzed in this study. For example, immunostaining using specific markers for the synaptic elements in the outer and inner plexiform layers (OPL and IPL, respectively; bassoon, red) and bipolar cells (PKC, blue), confirmed the otherwise normal structure of the retinas of GRF1-KO and GRF2-KO mice and showed that the presence of ectopic nuclei did not alter the photoreceptor synaptic pattern or the morphology and distribution of those cell types in the GRF2-depleted retinas (Fig. 1A–D).

**Ectopic nuclei in the PS layer of GRF2-KO retinas belong to cone photoreceptors**

Next, in order to identify the particular cell type housing the displaced nuclei observed in the PS layer of GRF2-depleted retinas, we performed immunolabeling assays using specific markers for different retinal cell types. These assays showed that the displaced nuclei always belonged to cells co-labeled with specific markers of cone photoreceptors including opsin blue (shortwave), opsin red/green (medium–longwave) and peanut agglutinin (PNA), (Fig. 1E,F). Furthermore, Toluidine Blue staining of semi-thin sections (Fig. 1G) confirmed the nature and integrity of the cell type harboring these ectopic nuclei, which showed the typical morphology, size and heterochromatin of regular cone photoreceptors (Carter-Dawson and LaVail, 1979). Finally, electron microscopic analysis of the ultrastructure and organelle content of the retinal cells harboring ectopic nuclei was also totally consistent with that of regular, morphologically normal retinal cone photoreceptors. As expected for regular cones, these cells were associated with Müller cells through adherens junctions present in the outer limiting membrane (OLM) (Fig. 1H) and also have an outer segment with a normal structure (Fig. 1I). These observations confirm the identity of the retinal cells harboring ‘ectopic’ nuclei as cone photoreceptors and show that, except for the abnormal nuclear location, they display normal cone structure, organelle content and morphology.

To further characterize this GRF2-dependent phenotype, we noted the location and quantities of ectopic nuclei present in the dorsal and ventral regions of the retinas of all four GRF genotypes under analysis here. For this purpose, we used dorso-ventrally oriented, semi-thin serial retinal sections stained with Toluidine Blue, and counted total number of ectopic nuclei present in sections corresponding to the temporal (T), central (optic nerve, ON) and nasal (N) areas of the retinas (Fig. 2A). Displaced nuclei appeared almost exclusively in GRF2-KO and GRF1/2-DKO retinas, but their distribution was not homogeneous, with a majority of the ectopic nuclei being preferentially located in the ventral region of the retina in these two genotypes (Fig. 2A). Interestingly, whereas the ectopic nucleus phenotype appeared to be exclusively linked to the absence of GRF2, we also observed that the GRF1/2-DKO retinas displayed higher numbers of displaced nuclei than single GRF2-KO retinas, suggesting that the additional absence of GRF1 also exerts some indirect effect, resulting in enhanced manifestation of this phenotype when GRF2 is already absent (Fig. 2A).

We also wished to ascertain whether the mislocalization of cone nuclei might have a negative impact on survival or viability of the cone photoreceptor cells harboring ectopic nuclei. Our immunohistochemical observations did not uncover any significant differences among the four different GRF genotypes with regards to the number of cells immunolabeled with specific antibodies against Ki67 or active caspase3 (data not shown), or the total number and local distribution of viable photoreceptor cells counted in the different regions of adult retinas of all four genotypes (Fig. 2B). Furthermore, our analysis of the immunostained sections of postnatal day (P)15 retinas also showed that the caspase3-positive cells detected in the knockout mouse retinas never corresponded to cones harboring ectopic nuclei.

**Manifestation of the ectopic nuclei phenotype during postnatal stages of photoreceptor development**

As the segments of the rod and cone photoreceptors in the mouse retina are known to start their development at P4 and are fully formed by P20 (Obata and Usukura, 1992), we wished to identify the precise developmental stage at which the ectopic displaced nuclei phenotype develops. Our initial observations of GRF1/2-DKO perinatal retinas between P5 and P15 showed a complete absence of displaced nuclei at P5 in any area of the retina (Fig. 3A), whereas a few scarce ectopic nuclei were found at P11 (Fig. 3B,D) and abundant nuclei, mostly located in the PS region of the ventral retina, were detected at P15 (Fig. 3C,D).

GRF proteins are postnatally expressed in the CNS of mammals (Feig, 2011; Fernández-Medarde and Santos, 2011) but no definitive data are yet available concerning their specific expression pattern in the retina. Thus, in order to determine whether there is any temporal correspondence between the patterns of expression of the GRF isoforms and the appearance of the ectopic nuclei phenotype, we analyzed retinas of WT control animals from P0 to adulthood by western immunoblot using specific antibodies against GRF1 or GRF2 (Fig. 3E). Our observations confirmed that the expression of both GRF proteins starts in the retina after birth and continues throughout adulthood. In particular, whereas GRF1 was first detected at P10, GRF2 was detectable at P4 and the expression of both proteins was also maintained during adulthood (Fig. 3E). In summary, our data documented that the displacement of ectopic nuclei, spherically to the OLM and into the PS area, occurs preferentially between P11 and P15, a time period where the GRF proteins are already present in the retina and GRF2, in particular, is already expressed at levels similar to those seen in adult retinas.

**Regular cone nucleus migration through the ONL is altered in retinas of GRF2-deficient mice**

During regular mouse retinal development, the nuclei of cone photoreceptors are known to undergo a final movement towards the OLM at the end of the so-called migratory phase, between P11 and P15 (Rich et al., 1997). As that timing is exactly coincident with the temporal window during which most of the ectopic nuclei were observed moving out of the ONL towards the PS area in GRF2-depleted retinas (Fig. 3), we hypothesized that cone nuclear
Fig. 1. See next page for legend.
Fig. 1. Ectopic nuclei in the PS region of GRF2-KO and GRF1/2-DKO adult retinas belong to cone photoreceptors. (A–D) Representative immunocytochemical labeling of retinal sections (see Materials and methods) from WT (A), GRF1-KO (B), GRF2-KO (C) and GRF1/2-DKO (D) adult mice. Regular photoreceptor nuclei, labeled in green, are packed within the ONL of all four different genotypes. Ectopic nuclei (arrows) located beyond the OLM (dashed line) and into the PS region are only visible in GRF2-KO and GRF1/2-DKO retinas. Normal synaptic terminals and bipolar cells are labeled with antibodies against bassoon (red) and PKC (blue), respectively. (E,F) Cells harboring ectopic nuclei in GRF1/2-DKO retinas express specific cone photoreceptor markers. (E) Simultaneous labeling of the perinuclear area of a cell harboring an ectopic nucleus (asterisk) with PNA (blue) and opsin blue (red). (F) Simultaneous labeling of the area surrounding an ectopic nucleus (asterisk) with PNA (blue) and opsin red/green (red). (G) Representative semi-thin section of a GRF1/2-DKO retina stained with Toluidine Blue showing that the morphology and heterochromatin content of ectopic nuclei corresponds to those of regular cone photoreceptors. (H,I) Normal ultrastructure of cells harboring ectopic nuclei in GRF1/2-DKO retinas. (H) Representative electron micrograph depicting the adherens junctions in the OLM (arrows) of a cell with an ectopic nucleus. The cytoplasm of these cells penetrates into the ONL (arrowheads). (I) Representative electron micrograph showing a normal outer segment (OS, arrows) in a cell with a displaced nucleus. In all micrograph panels the dashed lines mark the position of the OLM, asterisks indicate ectopic nuclei and the sceral part of the retina is oriented upwards. PS, photoreceptor segments; OS, outer segments; OLM, outer limiting membrane; ONL, outer nuclear layer; IPL, inner plexiform layer; INL, inner nuclear layer; GCL, ganglion cell layer; Mu, Muller cell. Scale bars: 50 µm (A–D); 10 µm (E–G); 1 µm (H,I).

movement might be deregulated in the GRF2-KO and GRF1/2-DKO retinas. To test this hypothesis, we carried out detailed measurements of the relative positions of the cones nuclei in the ONL of retinas of WT and GRF1/2-DKO mice precisely at P11 and of retinas of the four relevant genotypes at P15 – times corresponding to the beginning and the end of the terminal migration process. The position of each individual nucleus in the tissue sections analyzed was expressed quantitatively as the ratio between its distance (x) to the OLM and the total thickness (y) of the ONL (representative data and corresponding schematics shown in Fig. 4A,B). Our comparison of the cone nuclear movement ratios measured in multiple, different retinal sections obtained from different WT and DKO mice showed that the average value of the x/y ratio defining the position of the nuclei was already significantly higher at P11, and almost double by P15, in WT retinas compared with GRF2-KO or GRF1/2-DKO but not GRF1-KO mice (Fig. 4C). All in all, these data clearly showed that the migratory movement of cone nuclei towards the OLM was significantly altered in GRF1/2-DKO retinas as compared with their WT counterparts.

A more detailed analysis of the position of the migrating nuclei in all four separate genotypes was further facilitated by quantifying the number of nuclei located in each of 10 equal segments defined by dividing the ONL of each retina section analyzed (Fig. 4D). This analysis showed that at P15, when the physiological terminal migration of nuclear cones is completed (Rich et al., 1997), more than 60% of nuclear cones of GRF2-KO and GRF1/2-DKO retinas were already located within region 1, whereas less than 20% of nuclear cones in WT and GRF1-KO retinas had reached that position at that particular stage (Fig. 4D). Conversely, many more nuclear cones were found in areas 2–6 of the ONL of WT and GRF1-KO retinas than in the same areas of GRF2-KO or GRF1/2-DKO retinas. These observations indicate that the absence of GRF2 is specifically linked to excessive displacement towards the OLM of the position of the nuclei of cone photoreceptors of GRF2-KO and GRF1/2-DKO retinas, in clear contrast to the less displaced, more physiological position of these nuclei in WT and GRF1-KO retinas (Fig. 4D).

These data demonstrate that the altered final accumulation of cone nuclei in close proximity to (Fig. 4) or past the OLM (Figs 2 and 3) are cellular defects in the terminal movement of cone nuclei exclusively dependent upon the absence of GRF2 and that GRF1 is not directly involved in the generation of those phenotypes in mouse

Fig. 2. Number of ectopic cone nuclei detected in different regions of WT, GRF1-KO, GRF2-KO and GRF1/2-DKO adult retinas. (A) Bar plot showing numbers of displaced, ectopic nuclei counted in semi-thin sections of the ventral (blue) and dorsal (orange) regions of retinas of the indicated genotypes. N, nasal; ON, optic nerve; T, temporal. Predominant localization of ectopic nuclei in the ventral region of GRF2-KO retinas was observed. Total numbers of ectopic nuclei (means±s.e.m.) per section measured for each genotype were as follows: WT, 0.5±0.13 cells/section, n=3; GRF1-KO, 0.7±0.2 cells/section, n=3; GRF2-KO, 7.6±0.7 cells/section, n=4; GRF1/2-DKO, 11.4±0.8 cells/section, n=3. Statistically significant differences were observed comparing total numbers of ectopic nuclei of WT or GRF1-KO with GRF2-KO or GRF1/2-DKO (**P<0.001). Differences in the number of ectopic nuclei in GRF2-KO and GRF1/2-DKO were statistically significant (**P<0.05) only when comparing each specific retinal region (i.e. N, ON and T). The distribution of ectopic nuclei in GRF2-depleted retinas was not homogeneous, with predominant localization in the ventral area of the retina. GRF2-KO ventral area only (6.4±1.3) versus dorsal area only (2.4±0.4). **P<0.01. GRF1/2-DKO ventral area only (9.1±1.3) versus dorsal area only (1.6±0.25). **P<0.01. (B) Spidergram showing total numbers of photoreceptor nuclei in the ONL of retinas of the four GRF genotypes, identified by the indicated color codes. Numbers of photoreceptor nuclei were estimated by counting the number of nuclei/vertical row comprising the full thickness of the ONL each 250 µm from the optic nerve towards the dorsal and ventral part of the retina (regions 1–9). No photoreceptor degeneration was observed and no statistical differences were found for any of the individual genotypes. Data points are means±s.e.m., n=3.
retinas. Of significance for a finer definition of the GRF2-dependent cone photoreceptor phenotypes, it should also be emphasized that no differences were found among ventral, dorsal, nasal and temporal areas with regards to the distribution of the cone nuclei of GRF2-defective retinas that were abnormally accumulated in close proximity to, but not beyond, the OLM. This was in clear contrast to the situation with ectopic nuclei present in the same GRF2-deficient retinas, which were mostly identified as belonging to cones located preferentially in the ventral part of those retinas (Fig. 2). Taken together, our data indicate that when GRF2 is absent, a significant alteration or deregulation of the terminal movement of cone nuclei towards the OLM occurs, which results in abnormal accumulation of cone nuclei just under the OLM of all regions of the retina and, in some cases, even invasion of the OLM and protrusion of some of those nuclei deeper into the PS area, preferentially in ventral retinal areas.

Identification of additional signaling molecules participating in the process of GRF2-dependent cone nuclear movement

To try and uncover further mechanistic clues related to the above-described GRF2-dependent retinal phenotypes, we also performed various immunostaining assays using antibodies against specific downstream signaling molecules that could potentially interact with GRF2 and/or participate in signaling pathways relevant for the abnormal nuclear movement of cone photoreceptors observed in GRF2-depleted retinas.

For example, it has been reported that macromolecular complexes containing the PAR3 and PAR6 proteins play crucial roles in regulating apicobasal polarity, and relate nuclear movements migration in a variety of cellular systems (Solecki et al., 2004; Cadot et al., 2012; Sasaki et al., 2015). Interestingly, our immunocytochemical analyses of retinas of the four relevant GRF genotypes revealed that both PAR3 and PAR6 colocalize with β-catenin precisely at the OLM, the particular retinal layer marking the physiological limit for migration of cone photoreceptor nuclei in normal retinas that is also eventually trespassed by the abnormal ectopic nuclei frequently observed in GRF2-depleted retinas (Fig. 5). Strikingly, immunolabeling for the PAR3, PAR6 and β-catenin markers revealed the OLM as a continuous layer in the case of WT retinas (Fig. 5A) but showed clear discontinuities in GRF2-KO (Fig. 5B) and GRF1/2-DKO retinas (Fig. 5C), precisely in the vicinity of the same areas where displaced, ectopic nuclei are observed protruding and located into the neighboring PS layer (Fig. 5B,C). These observations clearly indicate that the integrity of the OLM, defined by the continuous distribution of PAR3, PAR6 and β-catenin, is compromised in the absence of GRF2.

The PAK proteins are known downstream effectors of the small GTPases Rac and CDC42, and have also been reported to play an essential role (upon activation by phosphorylation) in cytoskeletal reorganization processes regulating cell motility and morphology (Zhang et al., 1995). As Rac and CDC42 are also known potential targets for positive or negative regulation by the GRF GEFs (Calvo et al., 2011), we wished to examine the status of PAK1 and its activated, phosphorylated form, pPAK1, in the retinas of our control and GRF-knockout mice (Fig. 6). Thus, our parallel immunolabeling studies using antibodies against the unmodified, total PAK1 and against its active pPAK1 form revealed similar expression patterns within the ONL area of the retinas of all four GRF genotypes under study here (Fig. 6A). Thus, total PAK1 was expressed in all the photoreceptor cells, with the staining localized surrounding the nuclei throughout the ONL (Fig. 6A). However, labeling with antibodies recognizing active pPAK1 showed weak staining throughout the ONL, and strong labeling of a specific, small population of photoreceptor cells concentrated in the most
scleral part of the ONL (Fig. 6A, arrows). Strikingly, whereas the WT and GRF-knockout retinas showed similar patterns of pPAK1 immunolabeling within their ONL areas, GRF2-KO and GRF1/2-DKO retinas also displayed additional immunolabeling, revealing the presence of significant amounts of pPAK1 surrounding the nucleus of their ectopic nuclei (Fig. 6A, insets), suggesting that functional interactions between PAK and GRF proteins might also be involved in the process of cone nuclear migration.

Furthermore, consistent with our previous identification of cone photoreceptors as the cellular type undergoing altered nuclear movement and displaying ectopic nuclei in GRF2-depleted retinas, we also confirmed that the retinal cells expressing high levels of pPAK1 in the ONL (Fig. 6A) corresponded to that particular cell type by showing co-immunolabeling of the pPAK1-positive cells with antibodies against opsin red/green, a specific marker of cone photoreceptors (Fig. 6B).

Phosphorylated vasodilator-stimulated protein (pVASP) is an actin-binding protein that has been implicated in terminal migration of cone nuclei (Trifunović et al., 2010), and we were interested in...
analyzing its retinal pattern of expression in the context of the altered cone nuclear movement responsible for generation of ectopic nuclei. Double immunolabeling experiments of P15 retinas using antibodies against pVASP, and NSE as specific marker of cone photoreceptors (Rich et al., 1997) showed that pVASP is similarly expressed in fine, longitudinal cellular processes located within the

Fig. 5. OLM integrity in WT, GRF2-KO and GRF1/2-DKO retinas at P15. (A) Continuous staining of PAR6, PAR3 and β-catenin was observed along the OLM in WT retinas. Specific markers are indicated in each panel. (B,C) OLM integrity, demonstrated by the localization of PAR6, PAR3 and β-catenin, is compromised in the GRF2-KO and GRF1/2-DKO retinas where the nuclei are breaching the OLM. Immunolabel used is indicated in each panel. Figures presented are representative images from at least three retinas per genotype. PS, photoreceptor segments; OLM, outer limiting membrane; ONL, outer nuclear layer. Scale bars: 20 µm.
Fig. 6. Molecular identity of cells harboring displaced ectopic nuclei. (A) Subpopulation of photoreceptors containing high levels of the PAK active form pPAK (red, arrows) in the adult retina of the four relevant genotypes analyzed. PAK (green) is expressed in all photoreceptor cells in the ONL. Insets are higher magnifications of ectopic nuclei containing high levels of pPAK (red) in GRF2-KO and GRF1/2-DKO retinas at P15. Nuclei (blue) are labeled with Hoechst 33342. (B) The subpopulation of photoreceptors with high levels of pPAK (red) in the ONL of a WT retina (P11) correspond to cone cells. Cells with high levels of pPAK in the ONL (arrows) are colabeled with the red/green opsin (green). (C) Ectopic nuclei surrounded by PAR6 (red) and PAR3 (green) in a GRF2-KO retina (arrows). Sample genotype and immunolabel color are indicated in each panel. Representative images of a minimum of three retinas of each genotype. Scale bars: 50 µm (A); 20 µm (B, insets in A); 10 µm (C).
ONL of WT and GRF2-KO or GRF1/2-DKO retinas, (Fig. 7A–C). Strikingly, whereas in the WT only this weak staining was visible (Fig. 7A), in the case of the GRF2-KO and GRF1/2-DKO retinas, we also observed a small subpopulation of their cones that expressed very high levels of pVASP along their cellular bodies (Fig. 7B,C). Moreover, we also found significant accumulation of pVASP surrounding the displaced, ectopic nuclei found in the PS area of GRF2-KO retinas (Fig. 7C, left and right panels), suggesting that this protein is also implicated in the process of abnormal migration, conducting the cone nuclei towards the PS layer. Finally, because GRF2 downregulation has been reported to trigger CDC42-dependent, enhanced phosphorylation of PAK1 and myosin...
light chain 2 (MLC2), thus affecting cell morphology and movement capabilities (Calvo et al., 2011), we also analyzed the status of activated, phosphorylated MLC2 in the context of cone nuclear movement occurring in WT and GRF2-KO retinas at P15 (Fig. 7D,E). Our data showed that pMLC2 is significantly expressed in the OLM and PS areas of both WT (Fig. 7D) and GRF2-KO retinas (Fig. 7E), but also accumulated around the ectopic cone nuclei in the case of GRF2-KO (Fig. 7E) and GRF1/2-DKO retinas (data not shown).

GRF2-KO retinas show diminished cone responses in ERG assays
To determine whether the abnormal migration of cone nuclei observed in GRF2-KO retinas had any functional effect on the correct function of the retina, standard ERG protocols were used to record rod, cone and mixed responses in adult retinas. Strikingly, a significant reduction in the response of cone cells was observed in GRF2-KO and DKO retinas compared with WT retinas (Fig. 8). ERG wave amplitudes were clearly reduced in the GRF2-KO and DKO retinas in comparison to the controls (Fig. 8A). Average values for all ERG wave amplitudes showed statistically significant differences between the cone responses of WT mice and those of GRF2-KO or GRF1/2-DKO mouse cones (Fig. 8B, ‘b-phot’). These data strongly indicate that the lack of GRF2 has a crucial impact on the physiology of cone photoreceptor cells in the retina.

DISCUSSION
Our parallel analyses of retinas from mice of four relevant GRF genotypes (WT, GRF1-KO, GRF2-KO and GRF1/2-DKO) sharing the same genetic background have established a direct, unequivocal correlation between GRF2 depletion and the appearance of a novel, distinctive cellular phenotype in the retinas of adult mice. This phenotype involves the specific presence of abnormally localized ‘ectopic’ nuclei in the PS layer of the retinas of GRF2-depleted mice (GRF2-KO and Grf1/2-DKO). We demonstrated that these ectopic nuclei were preferentially located in the ventral areas of GRF2-KO (Fig. 7E) and GRF1/2-DKO retinas (data not shown).

Fig. 8. Electoretinographic responses of WT, GRF2-KO and GRF1/2-DKO retinas. (A) Representative standard ERG recordings of rod, mixed and cone responses recorded from WT and GRF2-KO mice. Amplitude measurement of the a and b ERG waves (b-scot, a-mixed, b-mixed and b-phot) was performed as shown by arrows. (B) Histogram plot of averaged ERG wave amplitudes (means±s.d.) from WT, GRF2-KO and GRF1/2-DKO mice. Statistically significant differences were found between WT cone responses and those of GRF2-KO or GRF1/2-DKO cones. (*P<0.05; ***P<0.001; Student’s t-test with Bonferroni post hoc; WT, n=7; GRF2-KO, n=8; DKO, n=6).
From the mechanistic point of view, the specific observation of cone nuclear mislocalization in GRF2-KO and GRF1/2-DKO retinas supports a direct link between the disappearance of GRF2 and the development of the observed, defective retinal phenotypes. Our search for cellular mechanisms responsible for the abnormal accumulation of cone nuclei in the PS area revealed that this phenotype is the result of a defective or deregulated process of nuclear migration through the retinal ONL layer (Rich et al., 1997) that occurs specifically in GRF2-depleted cone photoreceptors and causes accumulation of cone nuclei in areas abutting the inner surface of the OLM and eventual trespassing through this layer and protrusion of the nuclei deeper into the PS zone.

Little is known about the mechanism and regulation of the nuclear movements occurring in cone cells in the postnatal retina. Correct nuclear positioning is known to be essential for adequate function of a wide range of cells and organisms from yeast to mammals (Duper and Etienne-Manneville, 2011). In vertebrate neuroepithelia, the nuclei of precursor cells undergo movements along the apicobasal axis in a process called interkinetic nuclear migration (INM) that is synchronized with their cell cycle and cell fate determination mechanisms, thus giving rise to pseudostratified epithelia (Formosa-Jordan et al., 2013; Latasa et al., 2009). INM is also essential for correct development of precursor cells and stratified layers in the vertebrate retina during embryonal development (Bassett and Wallace, 2012; Cepko et al., 1996; Del Bene et al., 2008), but the mechanisms of cone nuclear movement, occurring postnatally in a process called the late migratory phase (Rich et al., 1997), are still poorly understood. In any event, our observations in this paper identify GRF2 as a crucial molecular player in this process.

Previous studies of neuroepithelial differentiation and retinal degeneration processes occurring in mouse models (Garcia Arguinzonis et al., 2002; Song et al., 2014; Trifunovic et al., 2010) or in humans (Jacobson et al., 2003; Lotery, 2001; Mehalow et al., 2003; Pow and Sullivan, 2007; van de Pavert et al., 2004, 2007) have provided useful clues regarding the cellular mechanisms and signaling molecules that might be relevant for the origination of the novel GRF2-dependent phenotypes described in this report. Consistent with those reports, our immunolabeling analyses identified structural disruptions of the OLM and alterations of the expression patterns of several signaling molecules that were closely associated to the defective cone nuclear migration phenotype observed in GRF2-depleted retinas. As many of these signaling molecules are potential targets or downstream effectors of GRF signaling, their study might provide clues to ascertain the exact molecular mechanisms responsible for the GRF2-dependent defects of cone nuclear movement described in this report. Nevertheless, we should also mention that, in contrast to most models of defective cone nuclear movement, where the cellular morphological alterations are usually accompanied by photoreceptor cell death (Pow and Sullivan, 2007; Song et al., 2014; Trifunovic et al., 2010), the cones of GRF2-KO retinas presented normal morphology and their survival was not compromised (at least until our analysis at 5 months of age), suggesting significant mechanistic proximity between GRF2 and control of cone nuclear movements, although further functional studies are needed to confirm this notion. Even if the survival of cone photoreceptors is not compromised in GRF2-depleted retinas, their defective ERG patterns clearly indicate that their functional role in the vision process is compromised, and it will be interesting to determine whether the reduced ERG responses are due to alterations in the synaptogenesis (Rich et al., 1997) of the cone cells in retinas lacking GRF2. It will also be interesting to determine whether this defective retinal cone ectopic phenotype has more dramatic effects on vision in humans, because the number of cones is almost double in human (5% of all photoreceptors; Curcio et al., 1990) compared with mouse retinas (2.8%; Carter-Dawson and LaVail, 1979) and the foveal region of the human retina, which is responsible for visual acuity, is cone-dominated.

As reported for CRB1-defective mice (Mehalow et al., 2003), our observations documented that the absence of GRF2 is intrinsically associated with loss of the integrity of the retinal OLM, as proved by our detection in GRF2-depleted retinas of discontinuous immunolabeling for PAR3, PAR6 and β-catenin, which are essential components of this retinal layer (Bulgakova and Knust, 2009). The structural and functional relevance of CRB1 for integrity of the OLM (Mehalow et al., 2003) might also help to explain our detection of higher numbers of ectopic cone nuclei in GRF1/2-DKO than in the single GRF2-KO mice, in view of our previous transcriptomic analysis showing significantly altered levels of CRB1 expression in GRF1-KO retinas (Fernández-Medarde et al., 2009). In any case, mechanistically, it remains to be determined whether disruption of the OLM layer is a direct or just an indirect consequence of the absence of GRF2 in mouse retinas.

Macromolecular complexes containing PAR6, PAR3 and CDC42 have been implicated in the regulation of apicobasal polarity and associated nuclear movements in various cellular systems including neuronal migration or myotube formation (Cadot et al., 2012; Solecki et al., 2004). In contrast to most models of retinal degeneration, where the defective localization of PAR and other polarity proteins affects the whole OLM (Alves et al., 2013; Cho et al., 2012; Koike et al., 2005; Mehalow et al., 2003), those defects were limited in the OLM of GRF2-KO and GRF1/2-DKO retinas to the very specific regions neighboring the ectopic nuclei located in the PS area and, in addition, the adherens junctions between photoreceptors appeared to be normal upon electron microscopy observation. In any case, consistent with the participation of macromolecular complexes containing the PAR3, PAR6 polarity proteins in generation of the GRF2-dependent retinal phenotypes reported here, our analyses of GRF2-depleted retinas detected significantly altered distribution of PAR and PAR6, not only at the level of their discontinuous presence in the OLM, but also of their specific accumulation surrounding the ectopic nuclei accumulated in the PS area of those retinas. Interestingly, the GRF proteins have been shown to directly bind to, and prevent activation of CDC42 (a member of those polarity macromolecular complexes), in experiments where downregulation of GRF2 led to a CDC42-dependent increase in the levels of ML2 and PAR1 phosphorylation (Calvo et al., 2011). Strikingly, consistent with these observations, our immunolabeling assays also uncovered significant accumulation of activated, phosphorylated pPAK, pMLC2 and pVASP in the bodies of cone photoreceptor cells and around the ectopic nuclei protruding into the PS region of GRF2-depleted retinas. Because Rac and CDC42 are known targets for positive or negative regulation by the GRF proteins (Calvo et al., 2011; Feig, 2011; Fernández-Medarde and Santos, 2011) and they have also been shown to exert stage-specific functions in the control of polarity in physiological neurogenesis and in tumorigenesis (Baschieri et al., 2014; Vadodaria et al., 2013), our observations support the notion that the absence of productive functional interactions between GRF2 and its potential downstream targets Rac and CDC42 in GRF2-depleted retinas might lead to subsequent alterations of the formation or structure of PAR-containing, polarity-related macromolecular complexes as well as abnormal activation of related signaling molecules such as PAK (Zhang et al., 2011).
Materials and methods

Animals

Handling and experiments with mice were carried out according to EU and Spanish guidelines for the care and use of animals in research. GRF1-KO, GRF2-KO and GRF1/2-DKO mice were generated as described (Fernández-Medarde et al., 2002; Font de Mora et al., 2003). WT controls and knockout mice were maintained in a C57BL/6 background and kept on a 12 h light:12 h dark cycle. All animals in this study were genotyped and confirmed as non-carriers of the rd8 mutation known to be present in C57BL/6N mouse strains (Mattapallil et al., 2012).

Tissue processing

Animals were anesthetized with isoflurane prior to euthanasia by cervical dislocation. The temporal part of the eye was marked and eyes were dissected out and fixed in 4% formaldehyde for 4 h at 4°C or in 2% formaldehyde for 2 h at 4°C. After fixation, eyes were washed with PBS and the cornea and lens were dissected out. Eyes were cryoprotected in two steps of 10% and 30% sucrose, embedded in TissueTek and frozen in liquid nitrogen. Dorso–ventral 16 µm sections were obtained in a cryostat and kept frozen at −80°C until used.

For semi-thin and transmission electron microscopy analysis, the eyes were marked in the temporal area prior to fixation in 2% formaldehyde and 2% glutaraldehyde for at least 24 h at 4°C. After fixation, the anterior portion of the eyeballs was removed, and the eyecups were post-fixed with 1% osmium tetroxide in water, dehydrated in ethanol and embedded in Embed 812 (EMS) resin. Semi-thin sections (0.5 µm) were stained with uranyl acetate and lead citrate and examined with a Zeiss Embed 812 (EMS) resin. Semi-thin sections (0.5 µm) were stained with 1% osmium tetroxide in water, dehydrated in ethanol and embedded and in Embed 812 (EMS) resin. Semi-thin sections (0.5 µm) were stained with Toluidine Blue. Ultrathin sections were mounted on copper grids and stained with uranyl acetate and lead citrate and examined with a Zeiss EM900 electron microscopy. Micrographs were taken with a coupled digital camera using ImageSP software (Syprogen).

Immunohistochemistry

Sections were washed with PBS, blocked with PBS, 0.1% Triton X-100, 5% BSA and 2% goat serum (GS) for 1 h at room temperature (RT). Primary antibodies were diluted in PBS with 0.1% Triton X-100, 2% BSA and 2% GS, overnight at 4°C. After washing in PBS, sections were incubated with secondary antibodies (all from Jackson ImmunoResearch) goat anti-rabbit Alexa Fluor 488 or Cy3, goat anti-mouse Alexa Fluor 488 or Cy3, donkey anti-rabbit Alexa Fluor 488 or donkey anti-goat Cy3 diluted 1:600 for 1 h at RT, washed with PBS and mounted with ProLong Gold anti-fading reagent (Life Technologies). In some slides, nuclei were counterstained with Hoechst 33342 (Life Technologies). The primary antibodies used in this study were: PAR3 (1:250, Millipore, 07-330), PAR2 (1:100, Abcam, 6022), p-MLC2 (1:300, Cell Signaling, 3675S), lecin from peanut-FITC (1:500, Sigma-Aldrich, L7381). A minimum of three retinas from three different animals of each genotype was always used to ensure data reproducibility.

Images were acquired with a Leica TCS SP5 confocal microscope with the pinhole set to 1 Airy unit and 200:0.7 NA, 40:1.25 NA and 63×1.4 NA immersion oil objectives. The proper laser lines, 405 nm, 488 nm and 561 nm were employed to excite Hoechst 33342, Alexa Fluor 488 or FITC, and Cy3, respectively. Images were acquired sequentially, first obtaining Hoechst 33342 and Cy3 labeling and then acquiring the Alexa Fluor 488 staining. Images were imported to ImageJ software (NIH) using the LOCI Bio-formats plug-in and minor adjustments of brightness and contrast were performed. For 3D reconstructions, optical planes were obtained with a step size of 500 nm. Stacks were imported to Imaged and the 3D viewer plug-in was used.

Photoreceptor cell death quantification

To evaluate the number of photoreceptor cells in the retina, dorso–ventral semi-thin sections stained with Toluidine Blue were used. Three retinas of each genotype at 3–5 months of age were used. The number of photoreceptor nuclei in a row from the OPL to the OLM were counted in the ONL every 250 µm, starting from the optic nerve in both the dorsal and ventral directions. Three central sections from each retina were used for this analysis.

Quantification of ectopic cone nuclei

Semi-thin sections of 0.5 µm thickness stained with Toluidine Blue were used to quantify and localize the ectopic nuclei in the adult retinas. Oriented serial sections were obtained from temporal, central and nasal areas. Central area was defined as the presence of the optic nerve head, for the temporal and nasal areas the boundary between dorsal and ventral regions was defined as the middle point of the section. For each area, the number of ectopic nuclei was counted in the ventral and dorsal portions of the retina. For each eye, ectopic nuclei were counted in three non-consecutive sections from each area and averaged. Three eyes of different animals 3–5 months old were used in the quantification. Quantification of ectopic nuclei in GRF1/2-DKO perinatal retinas was performed in 16 µm cryostat sections. Eyes were oriented as previously described and total number of ectopic nuclei was counted in two non-consecutive sections for each retinal area (temporal, central and nasal). Four retinas of each time point were used for this quantification. Statistical differences were analyzed by using Student’s two-tailed, unpaired t-test and Microsoft Excel software.

Quantification of cone nuclei positioning

To localize the cone nuclei in the ONL, dorso–ventral oriented serial cryostat sections of P11 and P15 control retinas, P11 and P15 GRF1-KO or P15 GRF2-KO retinas were used. Sections were immunostained with β-catenin to label the OLM and opsin red/green to label the cones. Confocal images were obtained at 20× magnification of the ventral and dorsal areas of the retina in a central position between the optic nerve and the ora serrata. Three images from each area (dorsal and ventral) from the temporal, central and nasal retina were used for the quantification. Because no differences were detected among the different regions, measurements from all regions were plotted together. The distance in µm from the cone nuclei center to the OLM and the thickness of the ONL was measured for every cone using ImageJ. The relative position of every cone to the OLM was calculated dividing the distance of the cone nuclei to the OLM by the total thickness of the ONL in that position. In that way, 0 indicates the position of the OLM and 1 is the position closest to the OPL. The total number of measured cones for each group was: control P11 (1572), control P15 (1362), GRF1/2-DKO P11 (1408), GRF1/2-DKO P15 (2104), GRF1-KO P15 (1153), GRF2-KO P15 (696). To better represent the specific changes in the cone nuclei localization at P15, the OLM was divided into 10 equal regions where region 1 is the closest to the OLM and region 10 is the closest to the OPL, and each nuclei was assigned to one of these regions according to its relative position. Statistical differences were analyzed by using Student’s two-tailed, unpaired t-test and Microsoft Excel software and error bars indicate s.e.m.
Western blot
Retinas were dissected out without retinal pigment epithelium and snap-frozen in liquid nitrogen and kept at −80°C until used. Retinas were manually homogenized in lysis buffer and boiled for 10 min. Samples were loaded on 7.5% polyacrylamide gels. Proteins were transferred to PVDF membranes and blocked with TBS with 3% non-fat dry milk and 1% BSA. Anti-GRF1 and anti-GRF2 rabbit polyclonal primary antibodies were used as previously described (Fernández-Medarde et al., 2002). Fluorescently labeled secondary antibodies (Life Technologies) were used and visualized in an Odyssey imaging system (Li-Cor). Three adult DKO retinas and 14 WT retinas, from P0 to adult, were used in these assays.

Electroretinogram recordings
ERG assays were performed as previously described (Mayor-Torregrosa et al., 2005). Briefly, mice were dark adapted overnight and flash-induced ERG responses were recorded after stimulation with a Ganzfeld stimulator. Rod, mixed rod and cone responses were measured in WT (n=7), GRF2-KO (n=8) and GRF1/2-DKO (n=6) animals.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
E.S. and D.J. designed the study. D.J. performed most of the experiments. C.L. and E.S. generated experimental reagents. E.S. and D.J. wrote the paper.

References


