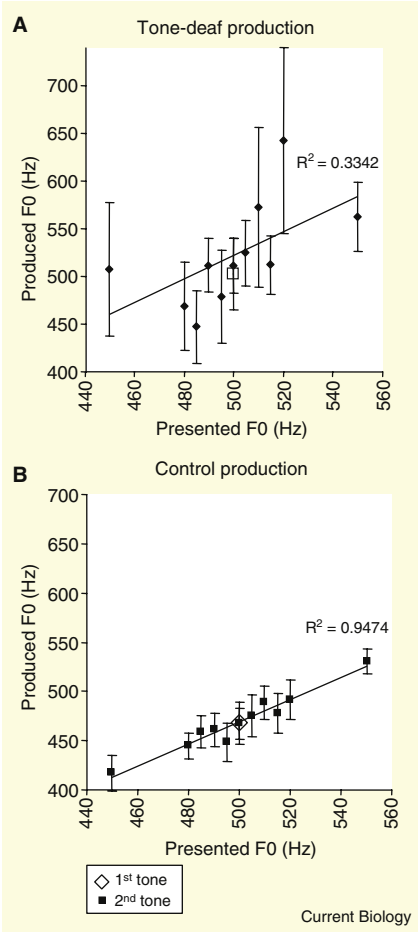

Figure 2. Variable pitch production by tone-deaf individuals.

(A) Mean fundamental frequencies of the two produced tones in tone-deaf listeners. The first tone had a target frequency at a constant 500 Hz, whereas the second tone ranged from 450-550 Hz. (B) Same as A in normal controls. While both groups show a significant positive correlation between target and produced fundamental frequency, the correlation is significantly lower in the tone-deaf group ($t(1,10) = 2.3$, $p = 0.046$) and variability in pitch production is higher for the tone-deaf group, as indicated by a t-test comparing standard error across different subjects producing the same pitch: $t(1,20) = 3.6$, $p = 0.0015$. Error bars indicate between-subject standard error.

Models of vocal communication generally involve interactions between the perception and production systems that allow the tuning of motor commands to achieve sound targets [3]. Our results shed further light on these models by indicating that the auditory pathways necessary for vocal performance are, to some degree at least, distinct from those necessary for conscious perception. The fact that tone-deaf individuals show no clear impairment in perceiving and producing speech provides further support for this conclusion. The distinction between auditory streams for production and perception demonstrated here may be analogous to separate visual streams for action and perception [9]. Further studies may aim to identify the precise neural correlates of this perception–action mismatch, and relate behavioral manifestations of tone-deafness to observed neurobiological anomalies in this unique population [10].

Supplemental data

Supplemental data are available at http://www.current-biology.com/cgi/content/full/18/8/R331/DC1

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FGF induces oscillations of Hes1 expression and Ras/ERK activation

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Many biological processes, such as circadian rhythms and somite segmentation [1], are regulated by molecular clocks. During somitogenesis, mRNAs for Notch signaling molecules, such as the Notch effector Hes1, oscillate periodically [1]. Here, we show that FGF stimulation induces the oscillatory expression of Hes1 in an ERK-dependent manner and also induces oscillatory activation of Ras and ERK activities. Our analysis demonstrates that oscillations in Ras/ERK activity require negative-feedback phosphorylation of Sos by ERK, suggesting that Ras/ERK oscillations could act as a novel molecular clock.

The oscillatory expression of Hes1 is triggered by serum stimulation in several cultured cell lines [2]. As FGF has been implicated in the regulation of somite segmentation [3], we examined whether FGF stimulation induces oscillation of Hes1 expression. Treatment of C3H 10T1/2 cells with bFGF induced the oscillation of *hes1* mRNA and Hes1 protein with a 2 hour cycle (Figure 1A and Figure S1 in Supplemental Data, published with this article online). To examine the potential involvement of the MEK–ERK pathway in triggering the oscillatory expression of Hes1, we examined the effects of U0126, a specific MEK inhibitor. Pretreatment with U0126 almost completely inhibited bFGF-induced oscillatory expression of Hes1 (Figure 1A). Moreover, pretreatment with another MEK inhibitor (PD98059) or expression of the MAPK phosphatase CL100/MKP1 suppressed the ERK activation and Hes1 oscillation (data not shown). DAPT, an inhibitor of γ-secretase, which cleaves Notch, did not prevent the Hes1 oscillation (data not shown). When U0126 was added to cells 110 min after FGF stimulation, the later rise in *hes1* expression was suppressed (Figure 1C). Unexpectedly, we then found that ERK phosphorylation, and therefore activity, oscillated in response

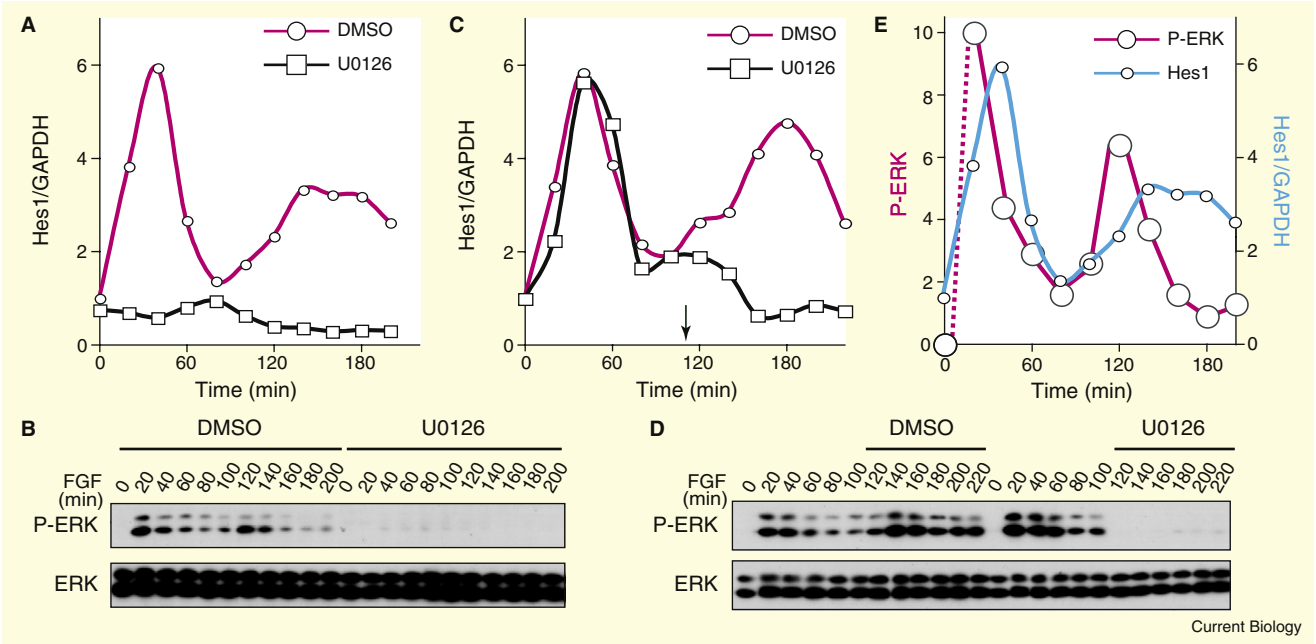

Figure 1. FGF-induced oscillations of Hes1 expression and ERK activation.

(A) *hes1* mRNA levels were determined every 20 min after bFGF (25 ng/ml) treatment of C3H 10T1/2 cells. Cells were pretreated 30 min before bFGF stimulation with DMSO (pink line) or with 20 μM U0126 (black line). (B) Cell extracts were subjected to immunoblotting analysis with antibodies against ERK and phosphorylated ERK (P-ERK). Three independent experiments gave similar results. (C) The effects of addition of U0126 (black line, final 20 μM) to C3H 10T1/2 cells at 110 min after bFGF treatment (arrow). (D) Again, cell extracts were subjected to immunoblotting analysis with antibodies against ERK and phosphorylated ERK (P-ERK). Three independent experiments gave similar results. (E) Comparison of the time course of Hes1 oscillation (blue line) and ERK activity oscillation (pink line).

to FGF stimulation (Figure 1B,D). The periodicity of this oscillation in ERK activity was roughly the same as that of Hes1 oscillation, with the ERK oscillation slightly preceding that of Hes1 (Figure 1E). ERK activity did not significantly affect the stability of Hes1 protein (Figure S2). ERK activation is therefore required for the FGF-induced oscillatory expression of Hes1 and oscillations in ERK activity may play a role in fine-tuning the Hes1 oscillation.

We then found that ERK activity also oscillated in FGF-stimulated NIH 3T3 cells (Figure 2A,B), with the peak activities being significantly dampened with time. The oscillatory expression of Hes1 was also induced by FGF treatment in NIH 3T3 cells (Figure S3). To investigate the mechanism underlying the oscillation in ERK activity, we then measured Ras activity. To our surprise, Ras activity (Ras–GTP) also oscillated after FGF stimulation (Figure 2A,B): in contrast to the oscillation of ERK activity, the peak Ras activities were not significantly dampened during the oscillation progression, whereas the trough activities were increased (Figure 2A). Ras activity also oscillated in FGF-stimulated C3H 10T1/2 cells (Figure S1). We confirmed that our assay method specifically recognizes Ras–GTP (Figure S4). Ras/ERK activities therefore oscillate after FGF stimulation and it is possible that these oscillations may be more striking when assayed at the single-cell level. To examine whether FGF receptor activity is required for oscillations in Ras/ERK activities, we added the specific FGF receptor inhibitor SU5402 to cells 70 min after FGF stimulation and observed suppression of later rises in Ras and ERK activities (Figure S5). We then examined changes in Ras and ERK activities in response to EGF. In HeLa cells, EGF stimulation induced oscillations in Ras activity (Figure S6). These data suggest that the sustained activation of receptor tyrosine kinase is required for oscillations in Ras/ERK activities and that a component(s) common to both FGF and EGF signaling pathways is involved.

As feedback inhibition should underlie oscillatory activation of the signaling pathway [4], we directed our attention to Sos, the guanine nucleotide exchange factor for Ras. ERK-dependent phosphorylation of Sos results in its dissociation from Grb2 and thus leads to Ras inactivation [5–8]. Immunoblotting analysis showed that the mobility-shifted bands of Sos protein, which reflect multiple phosphorylation of Sos [8], oscillated in response to FGF stimulation (Figure 2C). It appeared that Sos phosphorylation at 1–10 min resulted in Ras inactivation, and Sos dephosphorylation at 40–60 min led to Ras reactivation (Figure 2C). To examine whether ERK activation is sufficient for Ras inactivation, we used ΔB-Raf:ER cells (NIH 3T3 cells expressing the B-Raf kinase domain fused to the estrogen receptor ligand-binding domain) [9]. Induction of B-Raf activation (at –30 min) resulted in sustained ERK activation and Sos phosphorylation and led to almost complete suppression of FGF-induced Ras activation (Figure S7). These results suggest that ERK-mediated phosphorylation of Sos is a cause of Ras inactivation. In fact, when ERK activation was suppressed by U0126, Sos phosphorylation did not occur and Ras activity was sustained at high levels (Figure 2C). Moreover, our observation that the peak activity of ERK decreased with time and the trough activity of Ras increased in FGF-stimulated cells (Figure 2A,B) is also consistent with the above idea.

To determine whether ERK-mediated phosphorylation of Sos is required

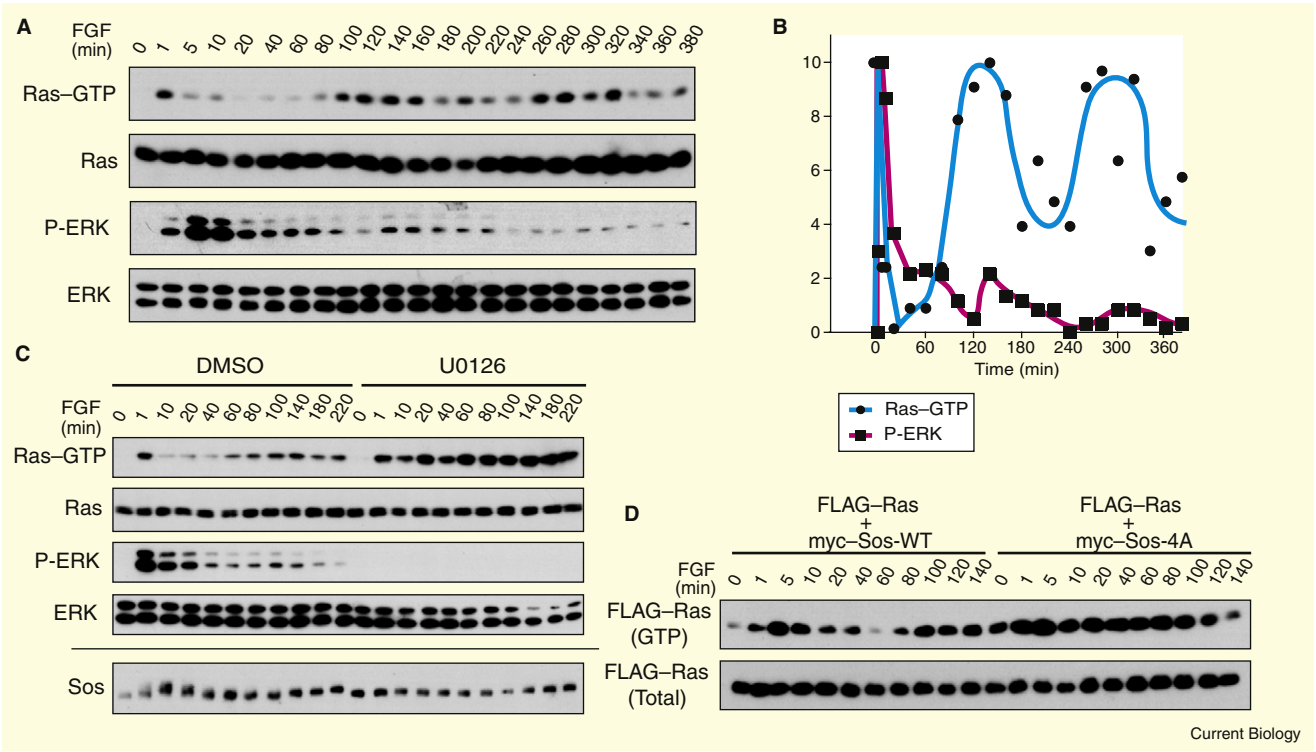

Figure 2. FGF-induced oscillatory activation of Ras and ERK.

(A) The levels of Ras–GTP, Ras (total), phosphorylated ERK (P-ERK) and ERK (total) were determined in NIH 3T3 cells stimulated with bFGF (25 ng/ml). (B) Quantification of the signals of Ras–GTP and P-ERK. (C) ERK-dependent phosphorylation of Sos leads to Ras/ERK inactivation in NIH 3T3 cells. The phosphorylation levels of Sos, in addition to Ras-GTP and P-ERK levels, were determined during FGF stimulation in the presence or absence of U0126 (20 μM). DMSO or U0126 was added 30 min before bFGF stimulation. Essentially the same results were obtained in two independent experiments. The upper four rows were from the same series and the bottom row from the other series. (D) Expression of myc–Sos-4A, but not myc–Sos-WT (wild-type), abolished FGF-induced Ras activity oscillations. NIH 3T3 cells were cotransfected with FLAG–Ras (250 ng) and myc–Sos-WT or myc–Sos-4A (750 ng). Cells were stimulated with bFGF (25 ng/ml) at time 0 and incubated for the indicated times. Three independent experiments gave similar results.

for the oscillations in Ras activity, we used a Sos mutant, Sos-4A, in which the four ERK phosphorylation sites are replaced by alanines [8], resulting in resistance to ERK-induced dissociation of Sos from Grb2 [8]. When myc–Sos-WT (wild-type) was co-expressed with FLAG–Ras, the Ras activity (FLAG–Ras-GTP) oscillated after FGF stimulation (Figure 2D). In contrast, when myc–Sos-4A was co-expressed, the oscillation in Ras activity was abolished, and high levels of Ras activity were maintained (Figure 2D), suggesting that the ERK-dependent, negative-feedback phosphorylation of Sos is required for generating oscillations in Ras/ERK activity.

In summary, our results demonstrate that growth factor stimulation induces oscillations of Ras/ERK activities. Although the physiological significance is uncertain at present, oscillatory Ras/ERK activity may act as a novel molecular clock.

Supplemental data

Supplemental data including experimental procedures and supplemental figures are available at http://www.current-biology.com/cgi/content/full/18/8/R332/DC1

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