C-RAF deficiency causes cochlear abnormalities and profound sensorineural deafness in the mouse

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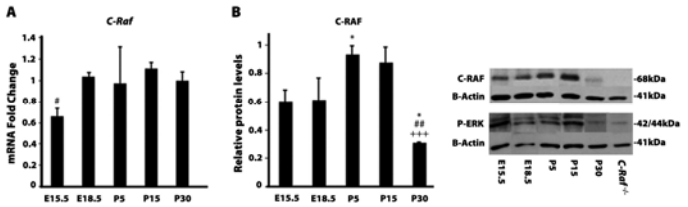
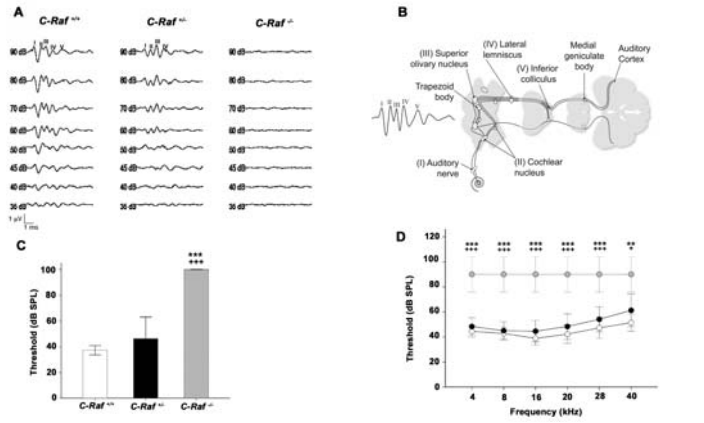
Insulin-like growth factor I (IGF-I) deficiency is associated with deafness[1]. Upon IGF-I binding to its high affinity receptor the RAF-MEK-ERK pathway is activated. RAF kinases are essential for cell proliferation, survival and differentiation during development and in the homeostasis of adult tissues[2]. RAF family proteins have redundant but also specific cellular and tissular functions. In the developing chicken inner ear, the activation of C-RAF and B-RAF are critical for otic neurogenesis and neuritogenesis[3]. To further study the role of RAF kinases in the auditory receptor, we have analyzed C-RAF mRNA and protein expression patterns in the developing mouse inner ear. Our results show that C-RAF is differentially expressed and that the protein is active and able to phosphorylate downstream substrates. To explore its functional relevance we have studied the phenotype of the *C-Raf*$^{-/-}$ null mouse. *C-Raf*$^{-/-}$ mutants present an all-frequency profound sensorineural hearing loss with a mean auditory threshold of 90 dB SPL. The study of the general cochlear cytoarchitecture indicates that the main structures and cell types have been formed, although the expression of molecules essential for hearing is altered. Thus the levels of the Kir4.1 potassium channel in the stria vascularis are reduced in the *C-Raf*$^{-/-}$ null mice, and the cellular localization of the transcription factor MEF2D is altered with respect to the wild type littermates. In summary, these results show that C-RAF is expressed in the developing cochlea and that its activity is essential for the onset of hearing.

Expression and activity of C-RAF kinase during inner ear development



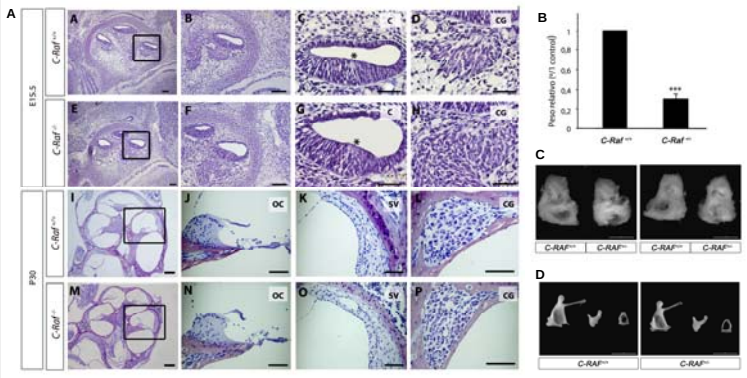
A) mRNA expression levels of *C-Raf* were analyzed by qRT-PCR at different stages. TBP RNA was used as the endogenous housekeeping control gene. The estimated gene expression was calculated as 2 –ΔΔCt. Results are expressed as mean ± SEM of at least 3 independent experiments performed in triplicate. Statistical significance was estimated by using Student's t-test: #P<0.05 versus E15.5. **B)** Inner ear lysates were analyzed by Western blotting, in order to determine the levels of C-RAF. A representative blot is shown and average values of densitometric measurements are plotted on bars. Results are given as mean ± SEM of 3 independent experiments. Statistical significance was estimated by using Student´s t-test: *P<0.05 versus E15.5, ##P<0.01 versus P15, +++P<0.001 versus P5 and *P<0.05 versus E15.5. The study of ERK phosphorylated (P-ERK), the RAF-MEK-ERK pathway activity indicator, showed that the cascade is activated in these same stages.

The *C-Raf* null mouse shows sensorineural deafness



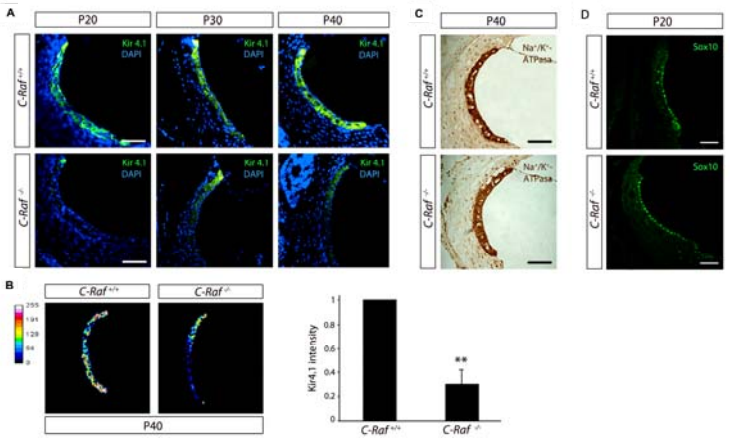
Hearing in C-Raf null mice: **A)** Representative ABR recordings for *C-Raf* wild-type, heterozygous and null mice. **B)** The typical ABR profile is formed for 5 waves that correspond to: I auditory nerve; II cochlear nucleus; III superior olivary nucleus; IV lateral lemniscus; V inferior colliculus. **C)** Average ABR thresholds for click stimulus of *C-Raf* wild type, heterozygous and null mice. Statistical significance was estimated by using Student´s t-test: ***P<0.001 versus *C-Raf*$^{+/+}$ and +++P<0.001 versus *C-Raf*$^{+/-}$. **D)** ABR thresholds in response to tone burst stimuli in *C-Raf* wild-type, heterozygous and null mice, the latter displaying severe sensoryneural hearing loss. Statistical significance was estimated by using Student´s t-test: ***P<0.001 versus *C-Raf*$^{+/+}$ and +++P<0.001 versus *C-Raf*$^{+/-}$.

Cochlear main structures and cell types are normal *in C-Raf* null mice



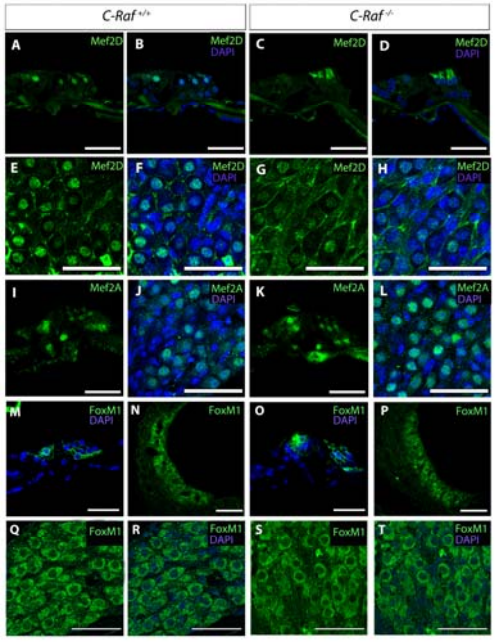
A) Hematoxylin and eosin staining of sections of the cochlea at E15.5 (A-H) and P30 (I-P) in wild type and null mice. There were no evident alterations in the animals studied. *C*, Cochlear duct; *O*, otic capsule; *OC*, organ of Corti; *CG*, cochlear ganglion; *VIII*, eighth cranial nerve; *TM*, tectorial membrane; *SL*, spiral limbus; *SV*, stria vascularis. **B)** Weight of *C-Raf* wild type and null mice, the latter presents a significant decrease (***P<0.001). **C)** The comparative study of the morphology of the inner ear of wild type and null mice did not show obvious differences in any of the structures. **D)** There were not differences either in the middle ear ossicles: malleus, incus and stapes among the genotypes.

C-Raf null mice show reduced levels of the potassium channel Kir 4.1



A) Morphology of the stria vascularis in *C-Raf* wild type and null mutant mice. Alterations observed included decreased expression of the potassium channel Kir 4.1 in the stria vascularis (green) at P20, P30 and P40. **B)** The quantification of the intensity of the immunofluorescent signal was carried out with Image J and is shown in the right panel. Data are expressed relative to the wild type value as mean ± SEM of at least 3 independent experiments. Statistical significance was estimated by Student´s t-test: **P<0.005 null versus wild type *C-Raf*. **C)** Immunostaining of the Na^+/K^+-ATPase channel in sections of *C-Raf* wild type and null mice showing no apparent differences. **D)** Immunofluorescence of Sox10 (green) in sections shows the normal presence of intermediate cells, melanocytes, of the stria vascularis in *C-Raf* null mice.
Melanocytes of the stria vascularis express Kir4.1 channel, this channel is essential to produce the cochlear endolymph and its deficit causes sensorineural deafness in mammals[4].

C-Raf null mice show altered expression of MEF2D but not of MEF2A and FOXM1



Immunofluorescence for the transcription factors MEF2D, MEF2A and FoxM1 (green) in sections of *C-Raf* wild type and null mice. Wild type mice showed MEF2D levels in the nuclei of inner and outer hair cells (A-B) and spiral ganglion neurons (E-F), while *C-Raf* null mice showed this transcription factor inactive in the cytoplasm (C, D, G, H). In contrast, the MEF2A is expressed in the nuclei of the inner and outer hair cells (I and K) and in those of the neurons of the spiral ganglion without showing differences between genotypes. Expression of FoxM1 did not show differences either between genotypes.
Cytoplasmic localization of MEF2D suggest a delay in the maturation of hair cells and neurons, probably due to the absence of C-RAF, and could therefore be one of the causes of sensorineural deafness[5].

CONCLUSIONS

1. *C-Raf* kinase is expressed, transcribed and it is active during mouse inner ear development.
2. Loss of *C-Raf* in mice causes neonatal all-frequency profound sensorineural hearing loss.
3. The general cytoarchitecture of the *C-Raf*$^{-/-}$ null mice cochlea is normal and presents all the cell types that compose the organ of Corti.
4. *C-Raf*$^{-/-}$ null mice cochlea show a reduced level of Kir4.1 but no of the Na K-ATPase. in the stria vascularis. Altered potassium homeostasis is sufficient to cause deafness.
5. *C-Raf*$^{-/-}$ null mice show cytosolic inactive MEF2D but not MEF2A or FoxM1 that show a similar cellular expression pattern than wild type mice.

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