Title
Allelic effects of RAS, BRAF and MEK on hair morphology.

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Allelic effects of *RAS*, *BRAF* and *MEK* on hair morphology
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I. Introduction

The Ras-MAPK (mitogen activated protein kinase) signal transduction cascade plays a crucial role in cell cycle control, differentiation, growth, and senescence [1,2]. However, germline mutations in genes that encode proteins in this pathway can lead to an increase in Ras-MAPK signal transduction, resulting in a spectrum of developmental abnormalities termed RAS/MAPK syndromes or 'RASopathies' [1,2].

Costello and cardio-facio-cutaneous syndromes belong to this class of congenital disorders and are associated with certain clinical phenotypes and mutations [1,2]. Gain-of-function mutations in *HRAS* alleles are associated with Costello syndrome (CS), and in *KRAS, BRAF*, *MEK1* and *MEK2* alleles, are associated with cardio-facio-cutaneous syndrome (CFC) [1].

RAS/MAPK syndromes are characterized by abnormalities in multiple organ systems, which may include craniofacial dysmorphology, hypertrophic cardiomyopathy and heart valve defects, neurocognitive impairment, short stature, musculoskeletal and ocular defects, and an increased risk of developing cancer [1,2]. Notable in the presentation of CS and CFC are cutaneous abnormalities, such as wrinkly skin and sparse, brittle, curly hair [1,2]. CS patients typically have hair which is curly, and either sparse or thick, with full eyelashes and eyebrows [1,3]. CFC patients are distinctive in their sparse, curly hair, which is wooly or brittle, with sparse or absent eyelashes and eyebrows [1,4].

Previous studies in mouse models reveal that RAS/MAPK gain-of-function mutations affect hair shaft production and morphology [5,6]. However, a detailed characterization of hair phenotypes in human CS/CFC has not yet been performed, partly because of the rarity of these syndromes.

II. Methods

Hair samples from seven Costello and CFC syndrome patients with a *HRAS*, *MEK1*, or *BRAF* allele mutation were obtained in collaboration with Katherine Rauen, UCSF director of the NF/RAS Pathway Genetics Clinic, and compared to five controls with varying degrees of normal hair curl. Hair shafts were analyzed for degree of hair curl, medulla, pigment distribution, thickness, shaft aberrations, cuticle, and cross sectional shape of hair shafts by light microscopy and scanning EM.

All light microscope images were taken using a DP71 (Olympus) camera mounted on a BX51 stereomicroscope (Olympus), unless otherwise indicated.

{Figure 1, Figure 2}

Degree of Hair Curl

Hair curvature was classified using a template from the *Atlas of Human Hair Microscopic Characteristics,* by forensic scientists Robert R. Ogle, Jr. and Michelle J. Fox [7]. Images of hair samples were taken using a dissecting microscope at 1x magnification.

{Figure 3}
Medulla, Pigment Distribution, Thickness, Shaft Aberrations
Hair shafts were mounted with Permount mounting medium on a glass slide, cover-slipped, and observed under light microscopy. For better visualization of the medulla in dark hair shafts, samples were bleached by treating with 10% H_20_2 at 4°C overnight or 30% H_20_2 at room temperature from 30min to several hours, until color was observed to fade. For more optimal contrast, samples were stained with Hematoxylin & Eosin after bleaching (see protocol below). Images were taken at 10x, 20x, and 40x magnifications.

Cuticle
Hair shafts were bleached and stained with H&E, as previously described. Images were taken under a Zeiss light microscope at 20x. A merged image was created from Z-Stack sections using Adobe Photoshop.

Scale casts were created using a method adapted from *Microscopy of Hair Part 1: A Practical Guide and Manual for Human Hairs,* by Douglas W. Deedrick and Sandra L. Koch [8]. Hair shafts were placed on top of a few drops of Permount mounting medium spread over a glass slide, and left to dry overnight. The following day, hair shafts were carefully removed, and the impression was photographed under light microscopy.

In addition, a NeoScope benchtop Scanning Electron Microscope by Nikon Instruments and JEOL was used to obtain images of hair shafts at 700x.

Cross-Sectional Shape
All sections >0.5 μm: Hair shafts were embedded with JB-4 Embedding Kit following Infiltration and Embedding procedures, sectioned using a tungsten carbide blade on a Leica 2125 Microtome, stained with Toluidine Blue or H&E (see below), and observed under light microscopy.

All sections 0.5 μm: Hair shafts were embedded with Embed-812/Epon812, sectioned on a microtome using a glass blade, stained with Toluidine Blue or H&E, and observed under light microscopy.

Hematoxylin and Eosin Stain: Hair shaft cross-sections were rehydrated with serial dilutions of EtOH to 70% and Milli-Q water. Hair shafts and cross-sections were stained with Hematoxylin, rinsed with Milli-Q and tap water, placed in 1% acid ethanol, and rinsed again with tap and Milli-Q water. Samples were then stained with Eosin-Y with Phloxine and dehydrated with serial dilutions of EtOH to 100% and Xylene. Hair shafts were placed on glass slides with Permount mounting medium (or Permount was added to cross-sections on slide), and samples were cover-slipped and let dry for at least several hours, usually overnight.

III. Results
{Table 1}

Hair curvature measurement template score classified all CS/CFC hair samples as tightly curled, consistent with common clinical descriptions.

{Figure 4}
Medulla, pigment distribution, and thickness were all within the spectrum of a normal tightly curled hair phenotype, and no shaft aberrations were observed.

{Figure 5, 6, 7}
No cuticle abnormalities were detected by any of the methods utilized—H&E staining, scale casting, or scanning electron microscopy.

{Figure 8}
In general, cross sectional shape was correlated with degree of curl: straight hair shafts had round cross sections, and as the degree of curl increased, cross sections became more elliptically shaped. Costello hair shafts seemed to show greater variety in shape, size, and diameter within a given hair shaft sample.

{Figure 9}
A more elliptical cross sectional shape was noted with CFC *BRAF* mutations, and a rounder shape with CFC *MEK2* mutations. This correlated with degree of curl measurements, with *BRAF* mutations having more tightly curled hair shafts. However, it remains to be determined if this observation could have any association with the report that patients with a *MEK1* or *MEK2* mutation show a less severe clinical phenotype than those with a *BRAF* mutation [9].

IV. Conclusions
The effects of activating mutations in *HRAS*, *BRAF*, and *MEK1* were examined on human hair. We found that all three mutations induced a tightly curled morphology.

Histologic and scanning electron microscopy analysis revealed the tightly curled phenotype of cardiofaciocutaneous syndrome hair to be indistinguishable from normal tightly curled hair. No differences were detected in Costello syndrome hair compared to normal tightly curled hair, except for greater variety of cross sectional shape; however, this analysis was limited to a single sample.

These findings suggest that the normal curled phenotype of human hair could be caused by increased RAS/RAF signaling. Although the alleles of RAS/MAPK syndrome patients vary in strength, curly hair development may respond after a signaling threshold is reached. It may be possible to predict specific phenotypes in humans experiencing excess or insufficient RAS/RAF signaling. Further efforts are ongoing to identify whether there are normal genetic variants in humans that affect RAS/MAPK signaling and hair morphology.

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Kit and Leica 2125 Microtome, and Eugene L. Cho**,** Bioscience Sales Specialist, Nikon Instruments Inc. for training and use of NeoScope scanning electron microscope.

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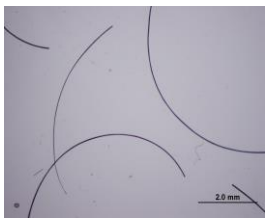
appendices

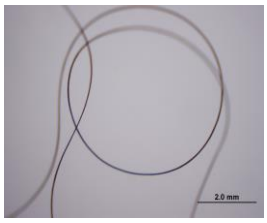
FIGURE LEGENDS
TABLES

Figure 1. Examples of Hair Specimens

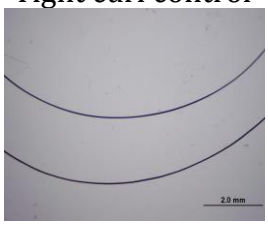
HRAS mutation



BRAF mutation



Tight curl control



Straight hair control

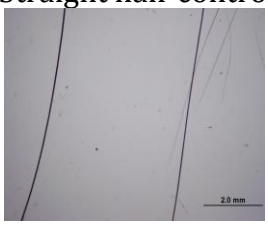
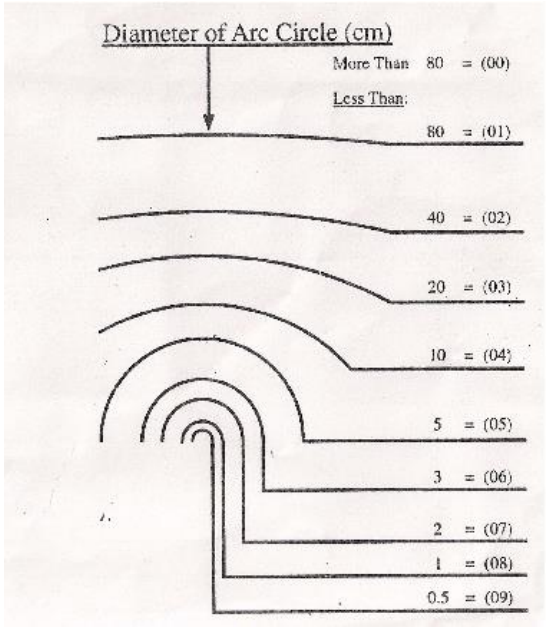


Figure 2. Hair Curvature Measurement Template

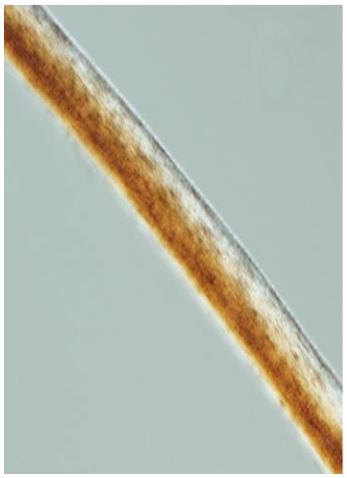


Hair Form	Degree of Curl
Tight Curl	< 0.5-2 Curls back upon itself to form circles
Loose Curl	3-10 Curls back upon itself to form circles
Wavy	Curvature changes direction Does not curl back upon itself
Curved	Slight curvature Does not curl back upon itself
Straight	≥ 80

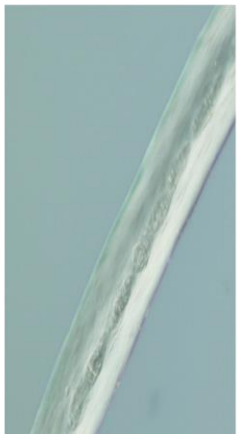
Atlas of Human Hair Microscopic Characteristics, Ogle RR, Fox MJ. 1999. CRC Press.

Figure 3. Light microscopy of hair shafts, examples of bleached and H&E staining

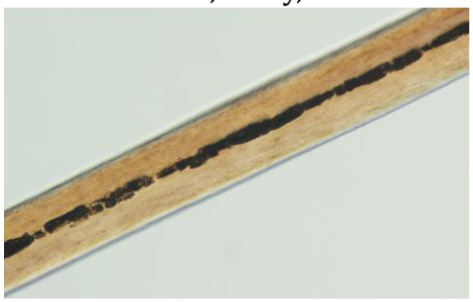
Costello, *HRAS*, 10x



Costello, *HRAS*, 10x, bleached



Control 4, Wavy, 20x



Control 4, Wavy, 20x, bleached + H&E

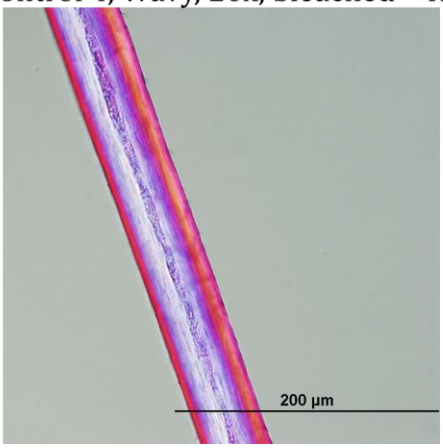


Table 1. Degree of Hair Curl and Classification

Sample, mutation	Degree of Curl	Hair Form
Costello, *HRAS*	1	Tight Curl
CFC (1), *BRAF*	0.5 - 1	Tight Curl
CFC (2), *MEK2*	2	Tight Curl
CFC (3), *BRAF*	<0.5 - 1	Tight Curl
CFC (4), *MEK2*	1	Tight Curl
CFC (5), *BRAF*	1	Tight Curl
CFC (6), *BRAF*	<0.5 - 1	Tight Curl
Control 1	1-2	Tight Curl
Control 2	2-3	Moderate Curl
Control 3	3	Loose Curl
Control 4	5-20	Wavy
Control 5	40-80	Straight

Figure 4. Light microscopy of hair shafts, Costello and CFC samples

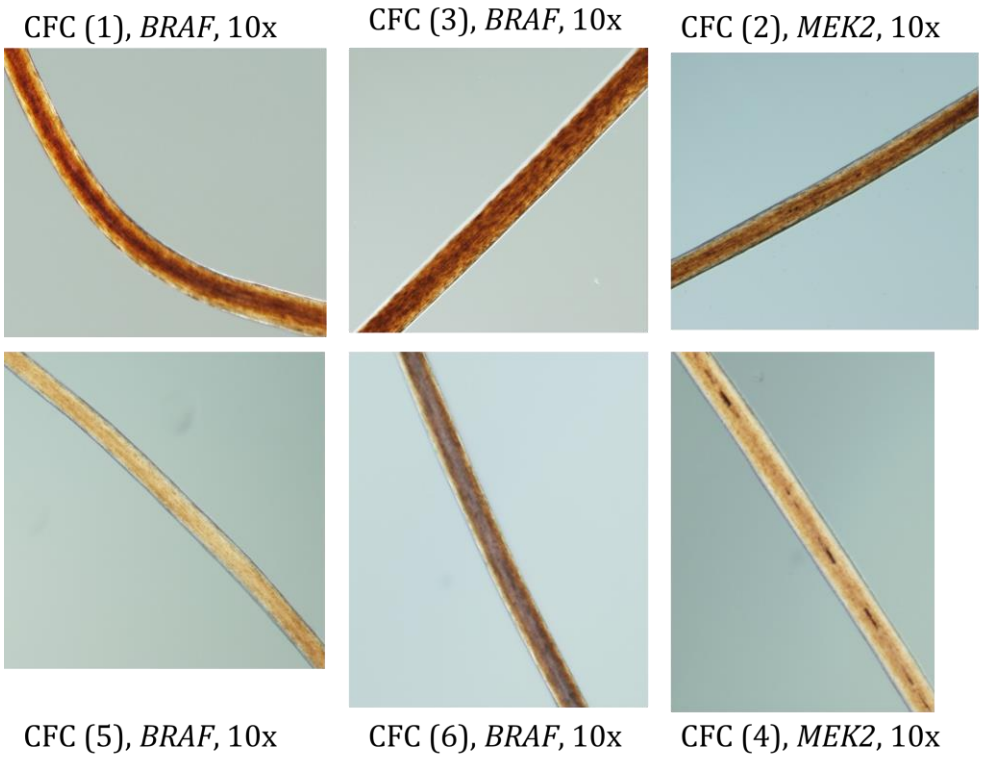


Figure 5. Merged Cuticle Pictures H&E 20x

Control 4, Wavy CFC (4), *MEK2*

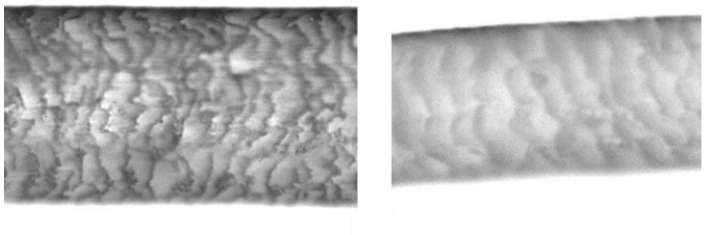


Figure 6. Scale Casts 20x

Control 2, Moderate Curl CFC (4), *MEK2*

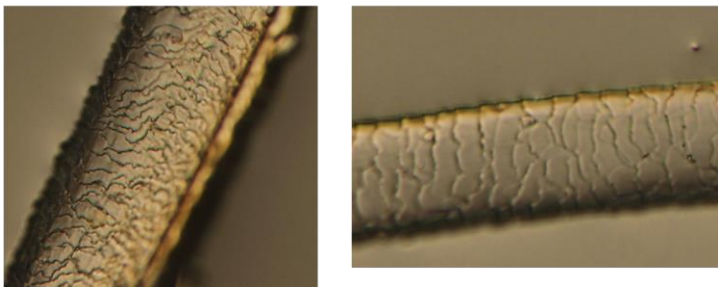


Figure 7. Scanning Electron Microscope 700x

Control 1, Tight Curl CFC (4), *MEK2*

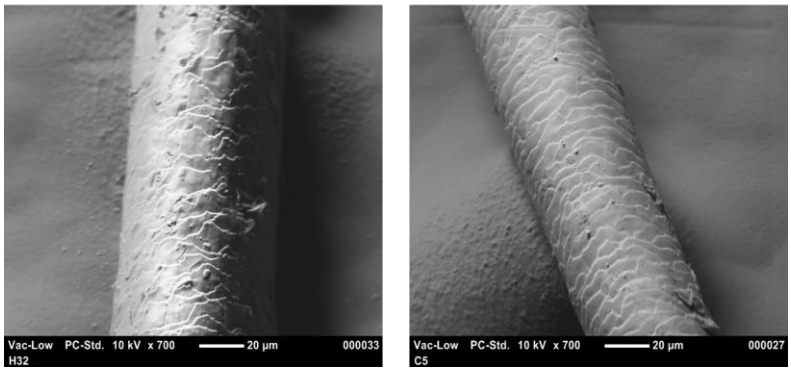


Figure 8. Cross Sections 0.5µm Toluidine Blue

Control 5, Straight | Control 4, Wavy | Costello, *HRAS*

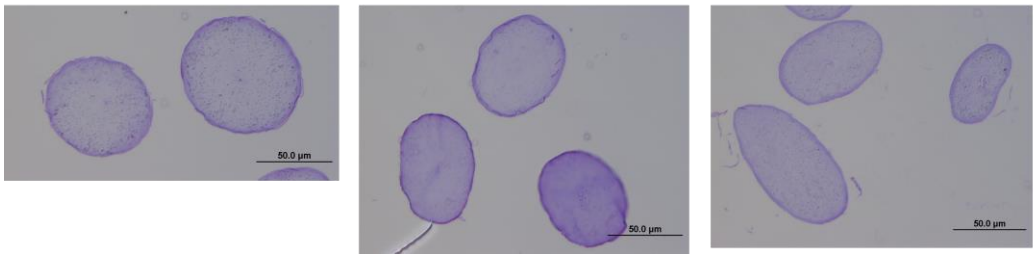
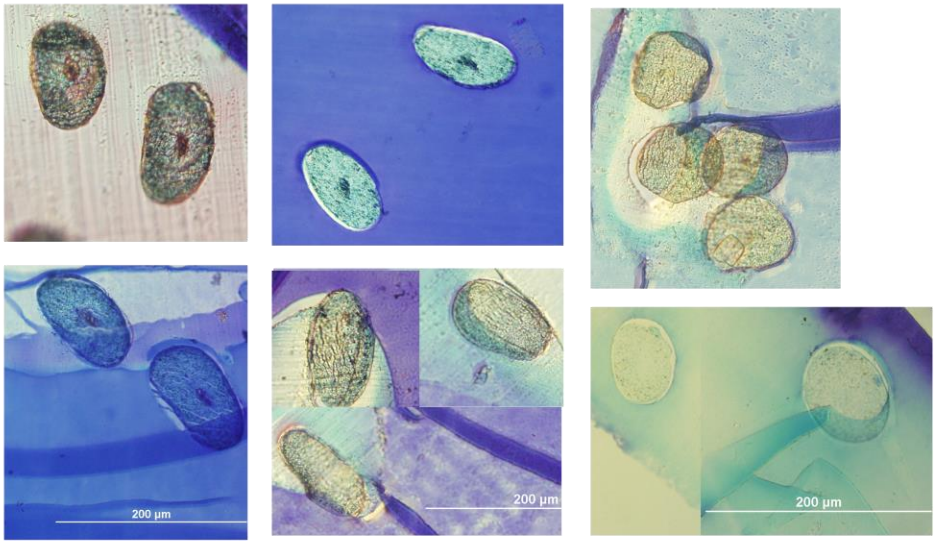


Figure 9. Cross Sections 2µm Toluidine Blue 20x

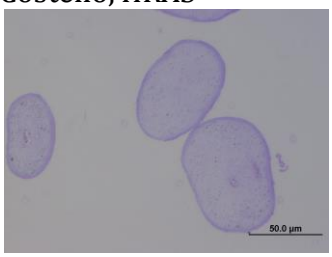
Control 1, Tight Curl | CFC (1), *BRAF* | CFC (2), *MEK2*



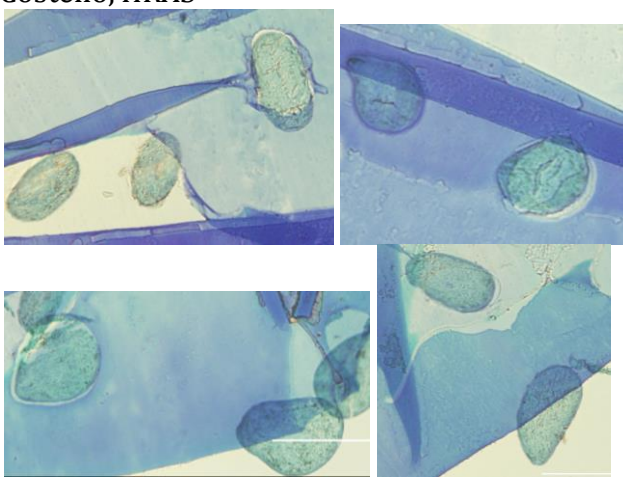
Control 2, Moderate Curl | CFC (3), *BRAF* | CFC (4), *MEK2*

Additional figures to consider including:
(Highlighting cross-sectional shape variability of Costello hair sample…)

Costello, *HRAS*



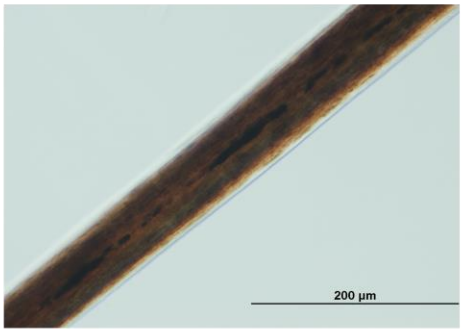
Costello, *HRAS*



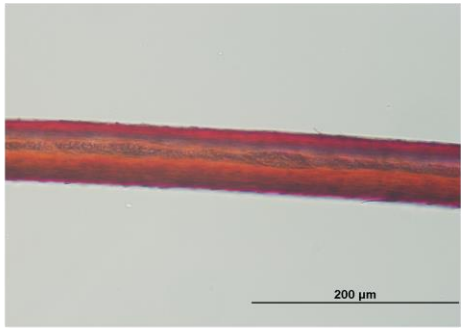
Example of light microscopy of controls (bleaching, H&E)

Control 5, Straight, 20x, unbleached

Control 3, Loose Curl, 20x, Bleached



Control 2, Moderate Curl, 20x, bleached



Control 1, Tight Curl, 20x, Bleached + H&E

KEY from poster to paper (change of sample names)

Costello, HRAS –C1		
CFC (1), BRAF –C2		
CFC (2), MEK2 –C3		
CFC (3), BRAF –C4		
CFC (4), MEK2 –C5		
CFC (5), BRAF –C6		
CFC (6), BRAF –C7		
Control 5 -H1	40-80	Straight
Control 3 -H4	3	Loose Curl
Control 2 -H9	2-3	Tight/Loose Curl (Moderate)
Control 4 -H29	5-20	Wavy
Control 1 -H32	1-2	Tight Curl

Control 1 -H32	1-2	Tight Curl
Control 2 -H9	2-3	Tight/Loose Curl (Moderate)
Control 3 -H4	3	Loose Curl
Control 4 -H29	5-20	Wavy
Control 5 -H1	40-80	Straight