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The Relationship Between Cutaneous Anthrax and Melanogenesis: A Toxic Affair

A Thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Biology

by

Jammal Abu-khazneh

Committee in charge:

Professor Ethan Bier, Chair
Professor Douglas Forbes
Professor Victor Nizet

2019

The Thesis of Jammal Abu-khazneh is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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University of California San Diego

2019

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This thesis, in full, is currently being coauthored with Annabel Guichard, Ph.D. Jammal Abu-khazneh was the primary author of this thesis.

ABSTRACT OF THE THESIS

The Relationship Between Cutaneous Anthrax and Melanogenesis: A Toxic Affair

by

Jammal Abu-khazneh

Master of Science in Biology

University of California San Diego, 2019

Professor Ethan Bier, Chair

The severe and often lethal disease Anthrax has been known since antiquity, and is caused by the spore-forming, gram-positive bacterium, *Bacillus anthracis* (*B. a*). After spores enter through an open wound, the host develops cutaneous Anthrax, which is identified by the development of typical black colored lesions, eschars. The name Anthrax itself derives from the Greek word for coal. Interestingly, the cause of the black eschars is not understood nor has their role in anthrax pathogenesis or host defense been adequately investigated. We set out to understand the cellular mechanism responsible for the formation of these black lesions, as well as understanding their role in disease progression. *Bacillus anthracis* secretes two toxic factors, Edema Factor (EF) and Lethal factor (LF), both paralyze the immune response in the early phase of infection and promote alarming symptoms in the later stages of the disease. Edema

Factor is a potent Adenylate Cyclase, that leads to an uncontrolled rise in cAMP concentration, and is responsible for edema; whereas lethal toxin is a zinc metalloprotease that cleaves MAPKK ultimately inhibiting cell growth and division. From experiments in *Drosophila melanogaster* transgenically expressing anthrax toxins, we find that cAMP signaling promotes pigmentation through a defined pathway mediated by Protein Kinase A (PKA). PKA is known to cause specific phosphorylation and activation of Tyrosine Hydroxylase (TH) the rate-limiting enzyme required for melanin synthesis. Examination of Tyrosine Hydroxylase in *Drosophila melanogaster* in response to EF expression shows a direct relationship with pigmentation and revealed novel phenotypes of Anthrax modeled in this insect system. We propose that a similar mechanism is at play in human cells, and that EF and LF may act similarly in melanocytes, potentially leading to uncontrolled melanogenesis in anthrax-infected skin tissues. Ultimately, this leads to the question as to whether these black cutaneous lesions are a product of host defense aimed at controlling the spread of the pathogen, or whether they are a part of *Bacillus anthracis* virulence strategy.

INTRODUCTION

Anthrax

Many of us vividly remember the 2001 anthrax scare that struck just a few days after the traumatic September 11 attacks. Envelopes containing microscopic spores were sent through in the US postal system, ultimately killing five, and sickening 17 more victims (Barstow). The bacterium that caused these casualties is well-known by scientists as *Bacillus anthracis* and its devastating outbreaks have been reported throughout history. Dating as far back as the Bible, the fifth plague of Egypt depicted in the book of Exodus is thought to be to anthrax according to the symptoms described (Ehrenkranz). Although mostly affecting herbivores, wildlife and cattle alike, the spore also infects humans, leading to early flu-like symptoms with widespread progression leading to death. Ancillary to this are black lesions on the skin. The name anthrax itself derives from “anthrakis,” the Greek word for coal, reflecting on an ancient and undeniable association between this disease and the color black (Kalamas).

Although these dark lesions are described in thousands of medical and scientific publications, as seen in **figure 1**, very little is known about the mechanism underlying their formation. Could they correspond to the accumulation of necrotic cells, or perhaps coagulated blood? Are they a byproduct of the infection, or a reaction of the assaulted organism trying to prevent the spread of the pathogen? Surprisingly, probing the scientific literature does not bring any answer to these questions. In this dissertation, we propose that the black pigmentation typical of cutaneous anthrax eschars is in fact caused by the production of melanin by host cells exposed to anthrax toxins. Using a fruit fly model system developed in our lab (Guicahrd), we showed that anthrax toxins

cause an array of dark pigmented phenotypes, which lead us to propose that the toxins induce host cells to produce an abnormally high amount of melanin. This further suggests that the well-known ubiquitous pigments that color hair and skin is also responsible for the long feared black eschars of the ancient disease anthrax.

Bacillus anthracis is a gram-positive spore-bearing bacteria that causes the disease anthrax. There are three known modes of infection: ingestion, inhalation, and cutaneous entry. The infectious spore can naturally be found in contaminated soil, and infect grazing animals by inhalation of the spores present in the dust, or ingestion through eating an infected plant or animal. An additional route, open wounds or even minor lesions that come in contact with anthrax spores are common entry routes for cutaneous anthrax infection, which is the most prevalent of the three modes of infection. At the site of cutaneous anthrax infection, skin lesions are associated with severe edema and black lesion formation. In the United States anthrax infections are not as prevalent with the current state of hygiene, animal vaccination and available antibiotics treatments; however, in third-world countries where vaccinations and antibiotic treatments are not as common, there are frequent cases of Anthrax infections (Kalamas).

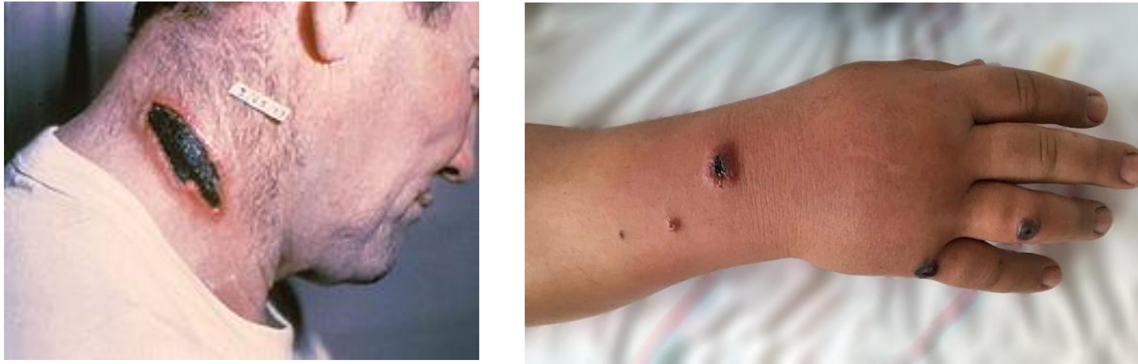

Figure 1: Cutaneous Anthrax skin lesions Cutaneous anthrax infected patients, with robust black eschars.

Bacillus anthracis only becomes active once inside the host, where the spores germinate, and the vegetative bacterium will secrete three proteins necessary to induce virulence; edema factor (EF), lethal factor (LF), and protective antigen (PA). These toxins are necessary for bacterial virulence (Guichard, Park). Together the toxins are responsible for paralyzing the immune response in early stages of infection, and causing systemic organ failure during late stages. PA facilitates the entry of the two other factors: it binds to the CMG2 and TEM8 receptors found at the surface of most cells and forms a pentameric pore, which allows entry of EF or LF. EF is a potent calmodulin-dependent adenylate cycle that leads to massive global production of cAMP (Brossier). This further keeps PKA permanently active, leading to sustained downstream phosphorylation of PKA targets, which ultimately causes prominent cell damage (Guichard, park). Additionally, the pathological amounts of cAMP prevent chemotaxis by white blood cells and alter their cytokine secretion resulting in severe tissue damage with multiple organ failure. Lethal factor is a zinc metalloproteinase that cleaves MapKinaseKinase (MAPKK or MEK), preventing cell division and causing

overall immune suppression **(Fig 2)**. In conjunction, when both EF and LF are present, the infection is often lethal unless treated with antibiotics, although such treatments may be inefficient if implemented too late, when massive amounts of the toxins have been irreversibly released into the bloodstream (Guichard).

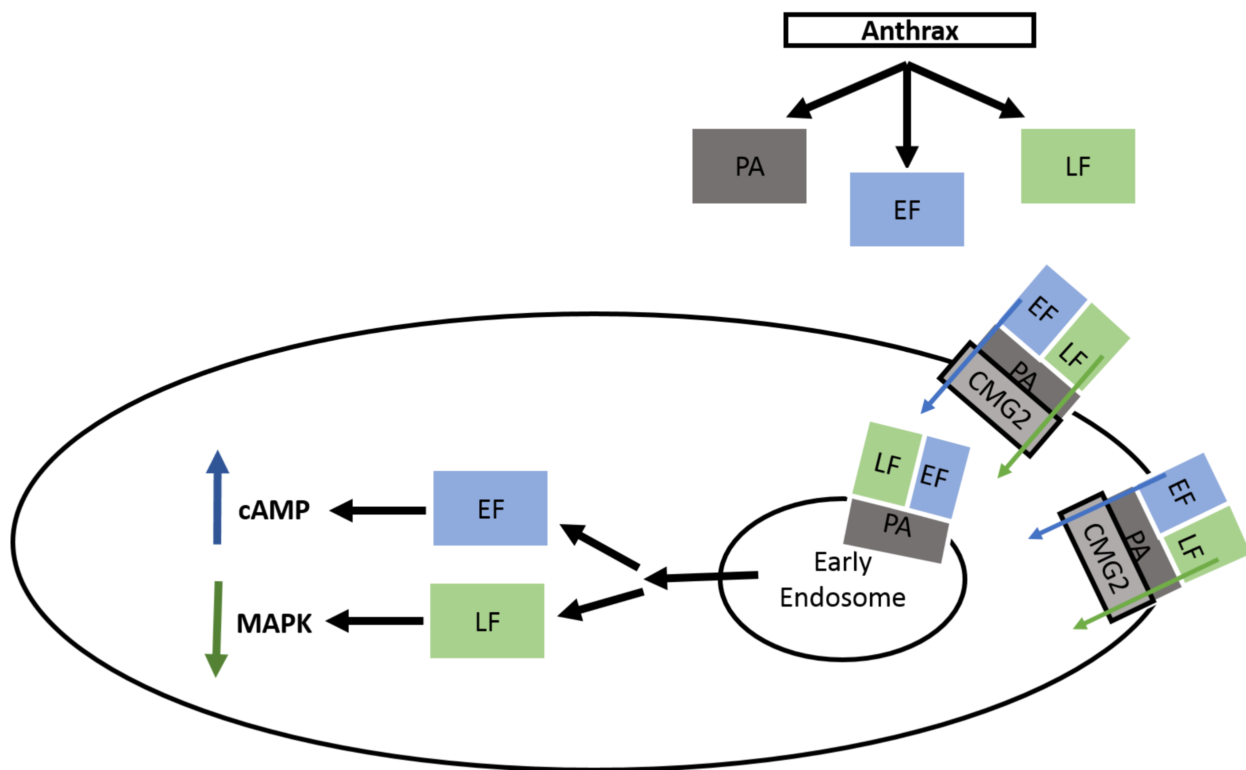

Figure 2: EF and LF's cellular entry, and mechanistic activity. Protective Antigen (PA) binds to the CMG2 receptor facilitating the entry of EF and LF, where both fuse to the early endosome. EF and LF separate, EF perpetuates an uncontrollable rise in cAMP leading to prominent cell damage, and LF cleaves MAPKK preventing cell division and attenuating the host-immune response.

***Cutaneous Anthrax and Melanogenesis*:**

Surprisingly, only one publication has addressed the relationship between melanogenesis and anthrax in any organism. A study performed by Koo et al. initially looking at using LF as a therapy for treating melanoma (skin cancer), coincidently found in mice, that EF and LF synergistically induce an elevation in pigmentation produced by

the melanoma cells. Furthermore, the group determined that LF was the main causative factor responsible for this increase, while EF alone had no detectable effect (Koo). The group proposed the hypothesis that MAPK negatively regulates melanogenesis. In support of this, since LF is known to regulate MAPK. Their study showed MAPK inhibitors yielded similar heavily pigmented phenotypes of increased melanogenesis when added to a B16 mouse melanoma cell line, the conventional cell-model system for studying melanogenesis.

This publication is the only report that pose a possible explanation for the cutaneous black lesions in anthrax. However, the authors did not propose a mechanism for the observed contribution of EF nor establish with certainty that melanin is produced by host cells in the context of an actual infection.

***Melanin*:**

Melanin is a polymer that is responsible for most of the dark pigmentation in the animal kingdom, it is synthesized in melanocytes, a specific cell type dedicated to the production of this pigment **(Fig 3A).** Melanin is found in the retina, neural crest and most, abundantly, within melanocytes (Sviderskaya). Melanin’s main function is thought to be to provide epidermal pigmentation that prevents DNA damage from UV radiation (Adenine Thymine dimerization), and is localized above the nuclei in keratinocyte cells, in the outer-most layer of the skin (Lin). This distribution represents a protective shield that absorbs sunlight, ultimately preventing DNA damage. It is well-known that sun exposure triggers melanin synthesis. Melanin is present in most organisms, signifying that this pigment is an extremely beneficial polymer and has functions across different

phylogenies (Robins). In mammals, melanocytes are derived from melanoblasts, which derive from the neural crest of the embryo (Sviderskaya). Although, melanin has been known for a long time it is a rich landscape for continued study.

Melanin is synthesized through a complex series of enzymatic reactions commonly known as the Raper-Mason pathway. The chemical process takes place inside melanocytes, more specifically in the melanosome, an organelle deriving from endosomes and responsible for melanogenesis (Borovanský). The melanosome contains a cavity where the reaction takes place: Tyrosine is converted to Dopa then to Dopaquinone through Tyrosinase, which is docked on the melanosome membrane. Tyrosinase is the rate-limiting enzyme responsible for pigmentation. Once dopaquinone is synthesized; the remaining chemical reactions are completed spontaneously without further enzymatic involvement, ending with two final hues of melanin: pheomelanin, a lighter colored pigmented melanin, and eumelanin, a darker colored pigmented melanin **(Fig 3B)** (Borovanský ,Slominski). Eumelanin is prevalent for those who have darker skin; whereas, lighter skinned individuals have less eumelanin and more pheomelanin (Borovanský).

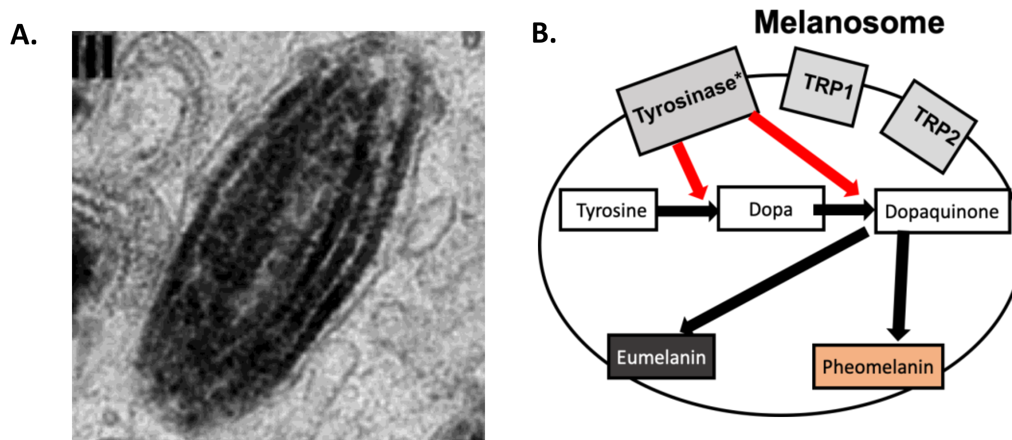

Figure 3: The melanosome, an organelle specific to melanocytes. (A) Low magnification imaging through electron tomography of early melanosomes. **(B)** Intra-organelle activity of the melanosome, highlighting key steps in the Raper-Manson pathway.

EF & Melanin in Drosophila melanogaster Model System:

*Drosophila melanogaste*r has been shown to be a genetically tractable model system suitable for investigation of anthrax's role in melanogenesis. This is because many of the enzymatic processes in melanin synthesis are genetically and biochemically well-understood in flies and humans, with seminal enzymes highly conserved between vertebrates and invertebrates (Guichard). Drosophila Tyrosine Hydroxylase, DTH, is a key component in melanogenesis and its amino-acid sequence shows 67% identity with human TH. Importantly, the PKA phosphorylation site Serine 32, present in human Tyrosine Hydroxylase is conserved with flies, suggesting that the mechanisms regulating its activity are also conserved (Vié A, Wittkopp PJ).

The commonly accepted pigmentation pathway in *Drosophila melanogaste*r begins with the conversion of Tyrosine to Dopa by DTH. Then dopa converted to dopa-melanin (a darker brown hue of melanin) by Phenol oxidase or converted to dopamine

through dopamine decarboxylase (DDC). A series of additional chemical reactions proceed ultimately ending with two other hues of melanin: dopamine melanin a light brown hue, and NBAD sclerotin (a yellowish hue) (**Figure 4**) (Vié A).

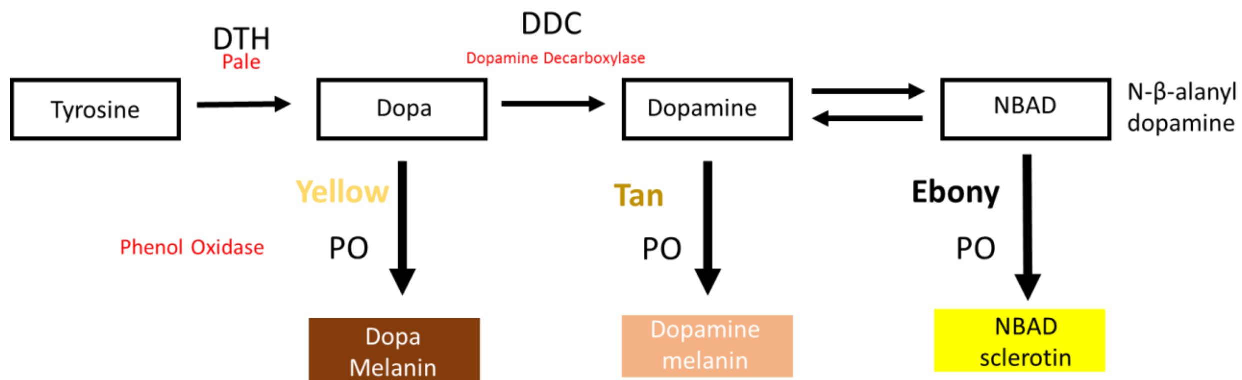

Figure 4: Melanin synthesis in Drosophila melanogaster. The biochemical pathway necessary for pigmentation synthesis begins with DTH converting Tyrosine to Dopa, and then branches out to different pigmentation hues, such as dopa melanin (black/dark brown), dopamine melanin (brown/tan), and NBAD sclerotin (yellow). The enzymes responsible for this conversion, are yellow, tan, ebony, respectively.

For our experiments, we used transgenic lines expressing LF or EF directly within the cell's cytoplasm, thus circumventing the need for PA-mediated endocytosis. Previous work conducted in the Bier lab established that EF and LF act on conserved cellular targets in Drosophila, such as PKA (EF), and MEK (LF), thus altering predicted developmental pathways. Further analysis revealed that EF and LF inhibit Rab11, a small GTPase located in recycling endosomes. Rab11 and the trafficking machinery essential for proper routing of various ligands and junctional components (Guichard A).

Melanocytes

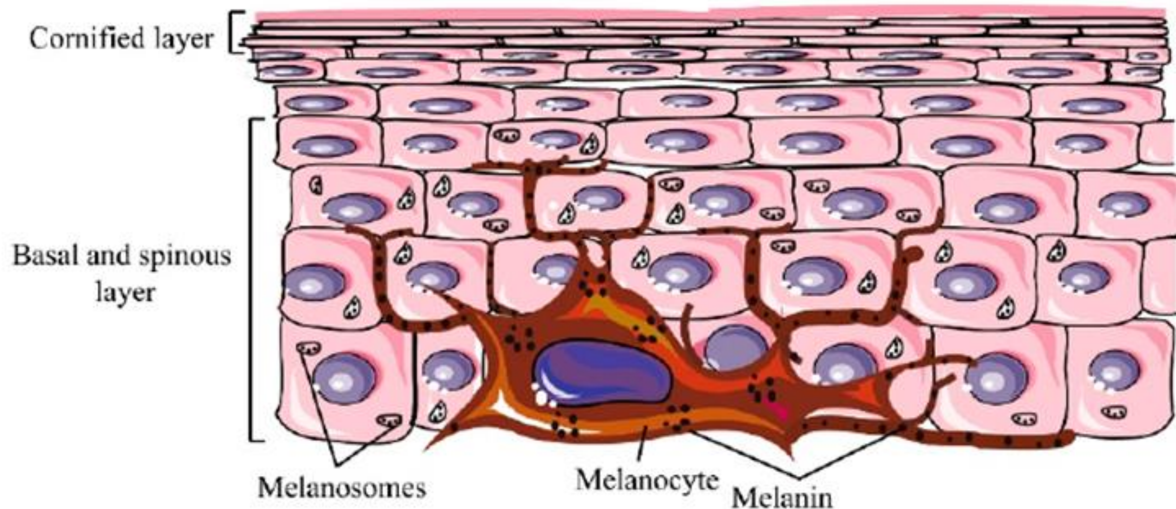

Figure 5: Melanocytes. Melanocytes are docked to the most inferior region of the epidermis, and most superior region of the dermis, adjacent to basal cells.

Melanocytes are melanin producing cells; anchored to the stratum basale, the most inferior region of the epidermis (Slominski). These cells have dendritic characteristics that help innervate the keratinocytes superior to them, with nearly 32 keratinocytes per every melanocyte illustrated above in **Fig 5** (Slominski, Park HY). The dendritic branches contain microtubules used by the vesicular trafficking protein, Rab27A, to transport melanosomes to the respective keratinocytes where they are further engulfed in a Rab11-dependent fashion and laterally docked to the periphery of the nucleus (Park HY). Once docked, melanin is released from the melanosomes and positioned above the nucleus to shield nuclei from UV radiation (Slominski).

Melanogenesis:

When keratinocytes absorb UV radiation or are stressed, they release Alpha melanin stimulating hormone (MSH), and Adrenal corticotrophin hormone (ACTH) which both induce melanin production. Both ligands bind to the GPCR Melanocortin 1 receptor, MC1R, found in melanocytes (Millington). Receptor activation leads to activation of Adenylate Cyclase (AC), which converts ATP to cAMP. The second messenger cAMP then activates PKA, leading to phosphorylation of various downstream targets. cAMP emanates two avenues to further induce melanogenesis; one of which, activates CREB (cyclic AMP response element binding protein), a transcription factor that facilitates the expression of the master regulator of melanogenesis: microphthalmia-associated transcription factor, MITF or MI. MITF then binds to several promoter sequences, leading to the expression of essential proteins necessary for melanogenesis: Tyrosinase, Tyrosinase related protein 1 (TRP1), and Tyrosinase related Protein 2 (TRP2) **(Figure 6).** The exact role of TRP1 and TRP2 is unclear; however, there is evidence that suggests that both proteins contribute to the stabilization of tyrosinase, the key enzyme in melanogenesis (Passeron, Yamaguchi). The upregulation of cAMP leads to the increase in expression and activity of Tyrosinase, ultimately promoting the elevation of melanin production through the Raper-Mason pathway (Flaherty, Passeron).

On the other hand, the phosphorylation of CREB also leads to the activation of MAP-kinase through a RAS exchange factor, which is exclusively found in melanocytes.

Prolonged activation of MAP-kinase leads to inhibition of melanogenesis through MAP-kinases' phosphorylation of MITF at serine 73. This phosphorylation is necessary for ubiquitination and degradation of MITF, which in turn stops melanogenesis **(Figure 6).** MAP-kinase can be viewed as a mechanism of retro-control used to regulate melanin production, ensuring an appropriate level of melanin synthesis (Yamaguchi).

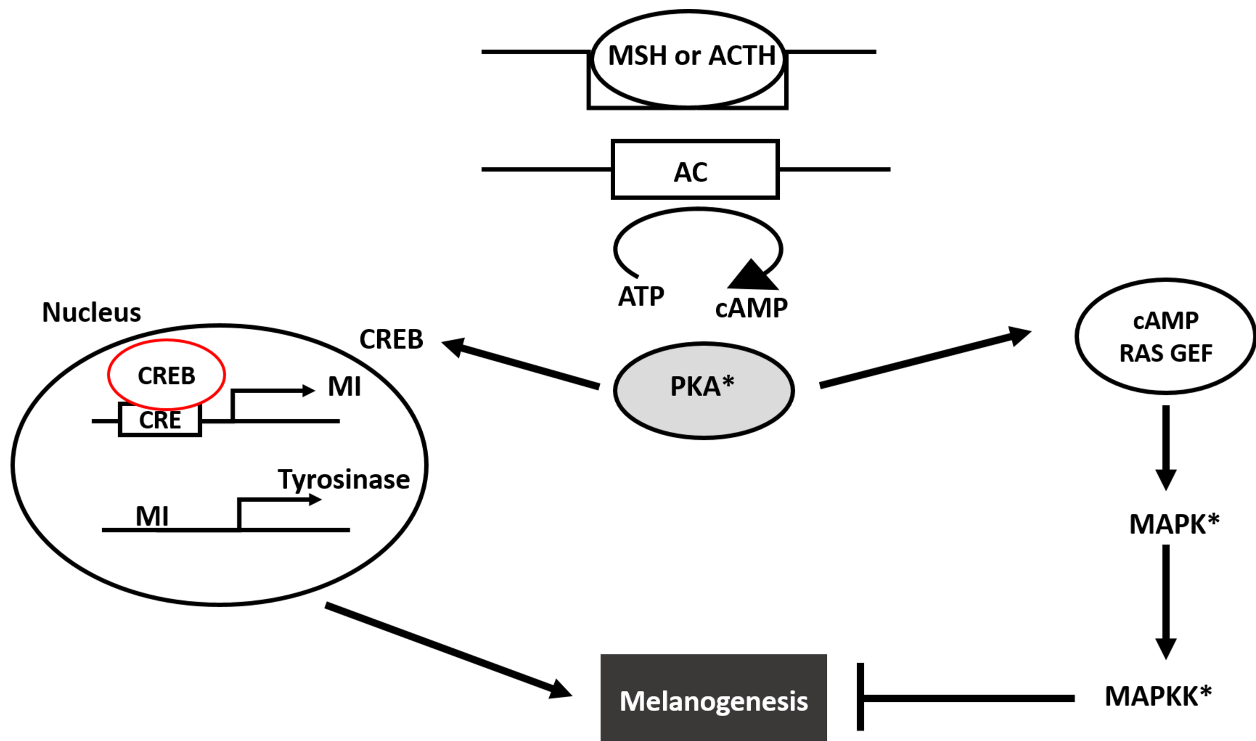

Figure 6: Regulation of melanogenesis in a melanocyte. Melanogenesis is regulated through the activation of Tyrosinase expression and activity by MI. Prolonged activation of MAPKK attenuate melanogenesis by ubiquitinating active MITF, promoting its degradation. Both pathways are used to synthesize a homeostatic balance of melanin.

Working Hypothesis of Anthrax and Melanogenesis:

The relationship between anthrax and melanogenesis is elusive, with minimal documented work addressing this relationship. We believe that anthrax toxins, the key virulence factors allowing bacterial growth and dissemination by causing symptoms in late stage infection, are also at the origin of the black eschars of cutaneous anthrax. We propose that EF and LF function synergistically to lead to an overproduction of melanin. EF triggers an uncontrolled rise of cAMP necessary to upregulate tyrosinase expression and activity; whereas LF cleaves MAPKK preventing a negative regulatory feedback of melanogenesis. Ultimately, we believe that these two factors concurrently act to stimulate melanogenesis through this mechanism.

METHODS

Drosophila Genetics:

All crosses were grown under standard conditions at 25 C with conventional cornmeal molasses agar media. UASwEF UAS-FLP/TM6 stock was used for EF expression, while LF was expressed by UAS-LF3X/FM7 stock. The GAL4 driver lines used included PPLGAL4, for expression in the fat body, and MS1096GAL4, for wing disc expression, causing wing phenotypes. Co-expression of UAS PLE or UAS-PLE RNAI with EF using MS1096 was established though conventional *Drosophila* genetics with three female virgins crossed to four adult males. w[*]; P{w[+mC]=UAS-ple.T}331f2 stock for UAS PLE expression, and y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS05881}attP2 was used for UAS PLE RNAi expression, both stocks were obtained from Bloomington, Indiana Drosophila Stock Center.

Drosophila Imaging

Flies were grown under standard conditions, and adult males 3 days old were placed in a 70% isopropanol solution for 2 days. Whole body images were taken on the GFP scope **(find exact name of scope)**. Wings were removed at their base and mounted onto glass slides with Canada Balsam solution used as an adhesive, then covered by a cover slip and left on a heating block for three to four hours. Wing images were taken on a **(get camera name),** and then further edited on Photoshop and analyzed through Image J.

Dissection, Mounting, and Immunofluorescence of Salivary Glands

Third instar *Drosophila* salivary glands were dissected under standard protocol in 1xPBS +, and then fixed in 4% formaldehyde, 1X PBS for 20 minutes. Glands were stained with the following antibodies: Anti-Tyrosine Hydroxylase, (orb224025 host-rabbit) and Anti-Phospho Tyrosine Hydroxylase (orb224025 host-rabbit) both followed by incubation with goat anti-rabbit Alexa Fluor 555-coupled antibody (Invitro-gen/Molecular Probes). In addition, glands were stained with DAPI (1/500).

Confocal microscopy was performed at UCSD Jacobs Medical Center using Leica (SP5), with photos further edited in Photoshop and analyzed with Image J.

hBMEC Staining

hBMEC were treated with indicated amounts of purified ET toxin (kindly provided by P. Jain) for 4 hours with a concentration of 0.2 ug/ml and stained using mouse anti-Tyrosine hydroxylase antibody (orb224025 host-rabbit) and Anti-Phospho Tyrosine Hydroxylase (orb224025 host-rabbit) both followed by goat anti-rabbit Alexa Fluor 555-coupled antibody (Invitro-gen/Molecular Probes) in independent experiments.

Confocal microscopy was performed at UCSD Jacobs Medical Center using Lecica SP5, with photos further edited on Photoshop and Image J.

RESULTS

Previous work in the lab established a *Drosophila* model for anthrax toxins (. Coding sequences for EF and LF were placed under the control of the UAS regulatory sequences (co-opted from yeast, Brand). When expressed in various tissues, EF and LF produced developmental phenotypes reflective of their know biochemical activities (over-production of cAMP and inhibition of MAPK signaling, respectively). This work also uncovered a novel activity of these toxins: the inhibition of Rab11-dependent trafficking (Guichard).

When initially investigating the various outcomes of toxin expression in *Drosophila* tissues, we encountered an unexpected phenotype when EF was expressed in the fat body, the *Drosophila* equivalent of the human liver. Adult males generated black patches consistently found on their abdomen, as well as patterned dark areas on their thorax. **(Fig 7A).** Males had larger robust black patches; whereas females had the same patches with weaker hues. Moreover, further investigation of EF expression in the wing disc illustrated a notable increase in coloration in all males and females, with males having remarkably darker wings. Twenty percent of the males had an overwhelmingly black wing **(Fig 7B)**. The conventionally known EF phenotype understood from previous work defines thickened wing veins and the loss of structural integrity with loss of wing margin; however, these black pigmented patches were something unanticipated, and never seen when modeled by any previous work. In essence, this helped establish a potentially novel functional role of EF, and that this virulence factor may contribute to the phenotype reminiscent of Cutaneous Anthrax in infected patients.

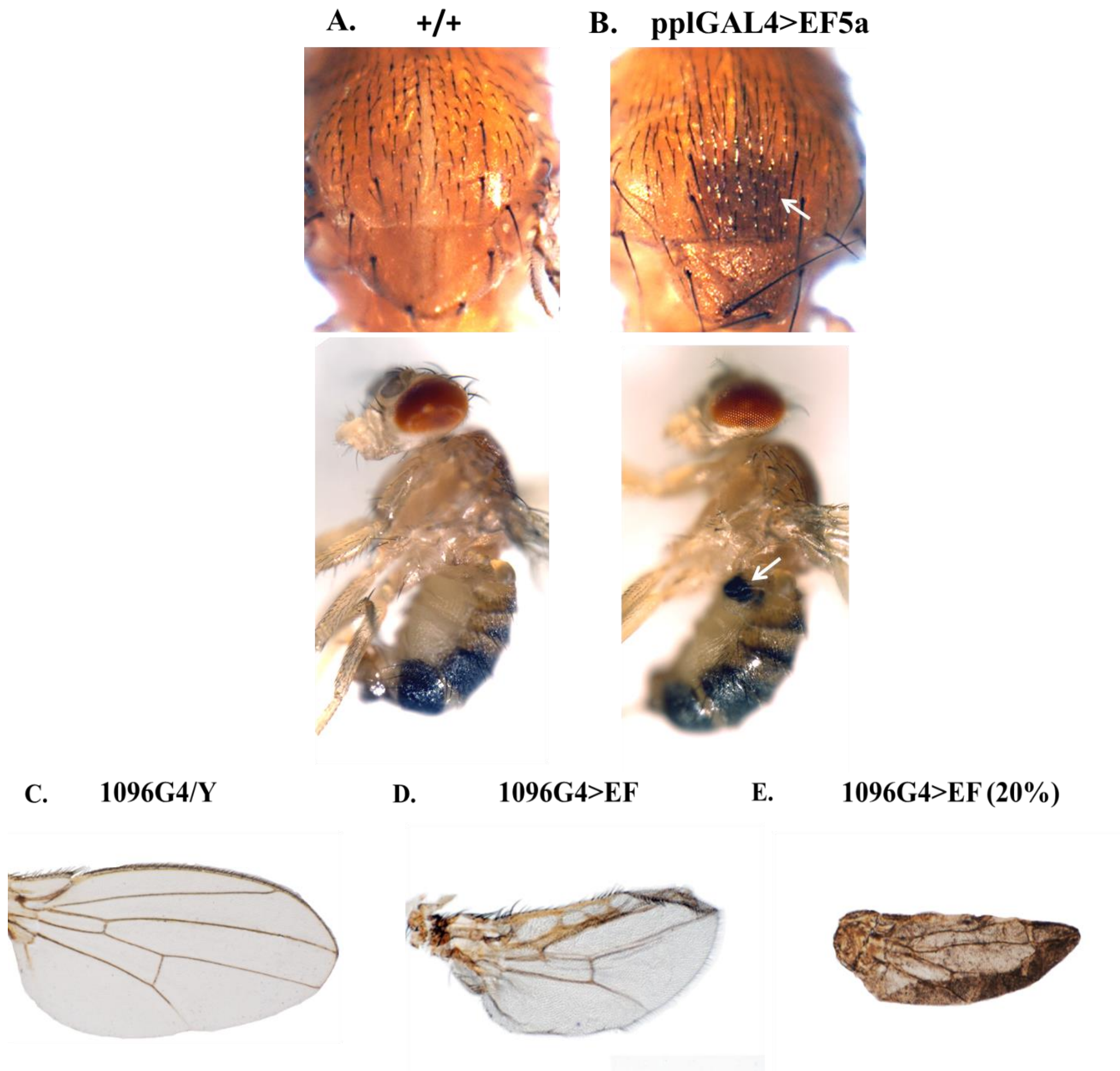

Figure 7: EF expression reminiscent of Cutaneous Anthrax. (A) Wild Type flies **(B)** 100% of Adult flies expressing EF in their fat body had black patches commonly found on their thorax and abdomen. **(C-E)** EF expressed in male wings through the 1096 G4 driver showed darker wing with the conventional EF phenotype: blistered wings, thickened wing veins.

Similar crosses using LF were also performed to establish whether parallel phenotypes similar to the elevated increase in pigmentation could be induced by LF. It's understood that EF and LF synergistically induce pigmentation when modeled on a murine system (Koo), and thus we hypothesized this phenomenon would be conserved

in our fly system. Unexpectedly, there was no change in wing color when expressed in the wing disc, as well as skin color when expressed in the fat body when LF was expressed alone or in conjunction with EF.

Previous literature has shown that PKA directly phosphorylates DTH at Serine32, which leads to an upregulation of expression and activity of this enzyme. This allowed us to then assemble the framework necessary to hypothesize a mechanism illustrating EF's relationship with DTH **(Fig 8)**, and how it may lead to the surge of pigmentation reminiscent of Cutaneous Anthrax's phenotype **(Fig 7).**

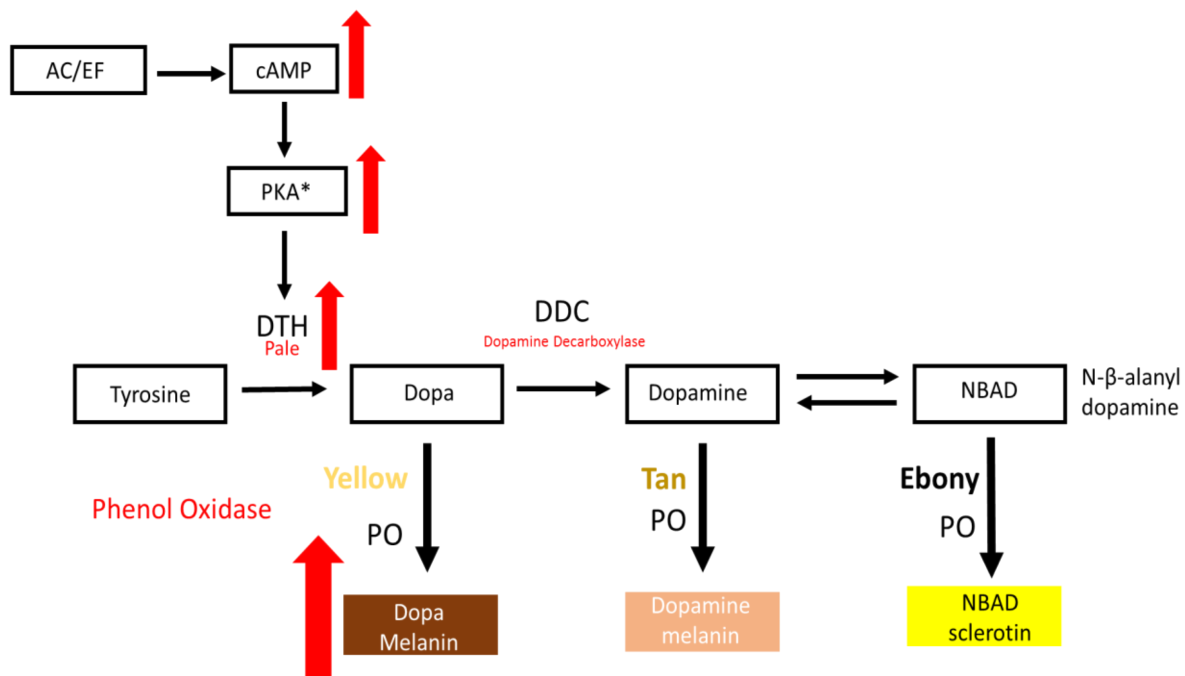

Figure 8: Hypothesis of *Drosophila* Tyrosine Hydroxylase's role in pigmentation. (A) Framework of *Drosophila* putative pigmentation pathway in the presence of EF. Induced cAMP levels indicated with red arrows further stimulate expression and activity levels of the rate limiting enzyme, DTH. Ultimately, pigmentation levels are surged.

Seeing that EF induces a pigmentation phenotype similar to that of Cutaneous Anthrax in humans, we investigated the effects of over-expression or blockage of DTH. The Pale, PLE, gene is the *Drosophila* homolog of DTH, and thus we first expressed the UAS PLE RNAi to knockdown DTH/PLE or, alternatively, we overexpressed DTH/PLE in the wing to identify phenotypes. When UAS PLE RNAi transgenes were expressed in the wing disc, the wings appeared entirely white and notably lost all signs of pigmentation **(Fig 9B)**. In addition, in the same region of the thorax where EF flies had black patches when expressed in the fat body **(Fig 7A)**, PLE RNAi flies showed a loss of pigmentation at a similar position on the thorax (not shown). In contrast, UAS PLE overexpression in the wings showed no change in wing phenotype: the wings looked very similar to wild-type with regards to both structure, and color **(Fig 9C)**.

With the modeled framework **(Fig. 8)**, we then set out to validate our hypothesized relationship between EF and DTH. We were interested in identifying any synergism or inhibitory effects between the two proteins. Through a two-generation cross-scheme, we co-expressed UAS PLE; EF and UAS PLE RNAi; EF in the wing disc and fat body in different flies. Interestingly, UAS PLE RNAi; EF showed the expected structural phenotype for EF alone: blistered wings with thickened wing veins, but also showed an entire loss of pigmentation very similar to seen when PLE RNAi alone was expressed in the wings **(Fig 9E).** Thus, with DTH/PLE knock down the pigmentation induced by EF was completely lost; the wings appeared depigmented analogous to PLE RNAi phenotype in the wings **(Fig 9B)**. In contrast, when PLE was overexpressed together with EF (UAS PLE;EF) the wings appeared significantly darker **(Fig 9F)**, than when EF **(Fig 9D)**, or UAS PLE **(Fig 9C)** was independently expressed in the wing disc.

In addition, the EF phenotype of thickened veins, loss of structural integrity was also present in the UAS PLE; EF fly line. **(Fig 9F)**. These observations thus support a model in which EF and DTH synergistically work together to induce greater pigmentation. This further indicates that EF is responsible for aberrant black pigmented areas, via activation of DTH, in our invertebrate model-system.

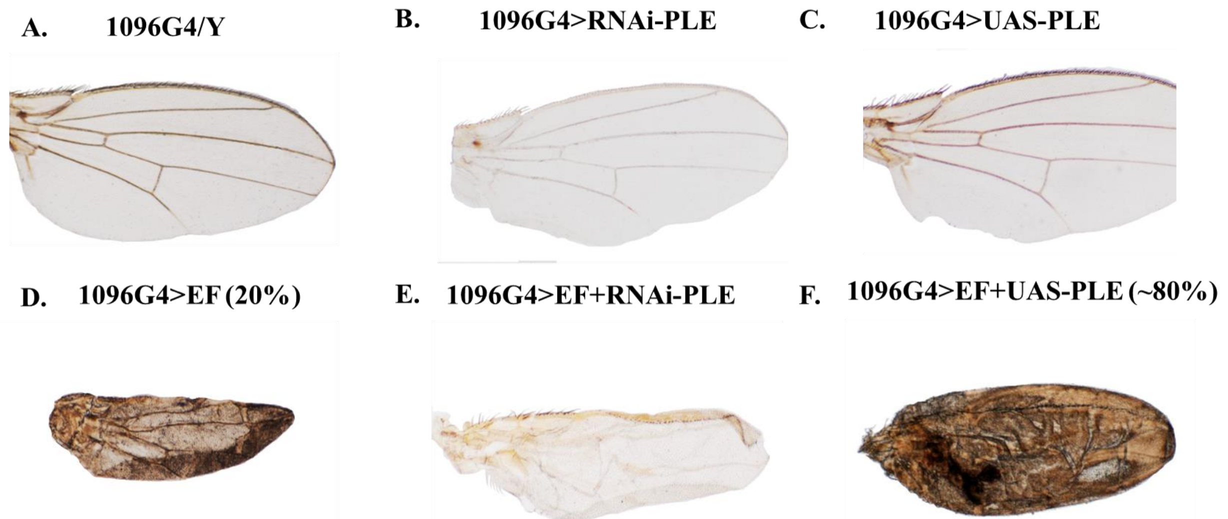

Figure 9: Drosophila Tyrosine Hydroxylase expression in the 1096 Wing Disc. (A-C) Males expressing an upregulation of DTH (UAS PLE) or the blockage of DTH (RNAi-PLE). **(B)** RNAi-PLE resulted in a completely albino wing, with a loss of pigmentation. **(C)** Overexpression of PLE (UAS PLE) remained similar to WT, 1096G4/Y). **(E)** The co-expression of RNAi-PLE with EF showed a mix of expected phenotypes: a completely albino wing with the loss of structural wing integrity and thickened wing veins. Overexpression of PLE Wings **(F)** were significantly darker when EF and excess PLE (UAS PLE) were co-expressed together, with notable black patches found on the wings.

To confirm that the wing phenotypes shown were specific to PLE and PLE RNAi, we examined the cellular levels and distribution of DTH in wild-type animals or in response to EF. We chose the salivary gland to assess this distribution because of their polytene chromosomes, which gives high overall protein expression. Moreover, the salivary gland cells are much larger in size relative to other cells in *Drosophila* allowing the enzymes to be more easily seen when stained **(Figure 10**).

When we stained for DTH wild-type (WT) male flies, there was minimal signal throughout the cells, with some granular signal at the plasma membrane, potentially at the cellular junctions. In addition, there was a notable amount of signal in the nuclear periphery outlining the nucleus, as well as some weaker signal in the cytoplasm **(Fig 10A)**. Expression of PLE RNAi illustrated a complete a blockage of DTH expression globally, with the minimal peripheral nuclear signal seen in WT suggesting this signal could potentially be artifact or residual PLE expression **(Fig 10B)**. When DTH/PLE was overexpressed (UAS PLE), there was a large increase in DTH expression across the plasma membrane, cytosol, and around the nuclear periphery **(Fig 10C**). In the presence of EF, expression there was also a noticeable increase in DTH levels in or around the nucleus as well as in the cytoplasm **(Fig 10D)**. These immunohistochemical findings help reinforce our conclusion from wing phenotypes and the hypothesized role EF plays in melanin production.

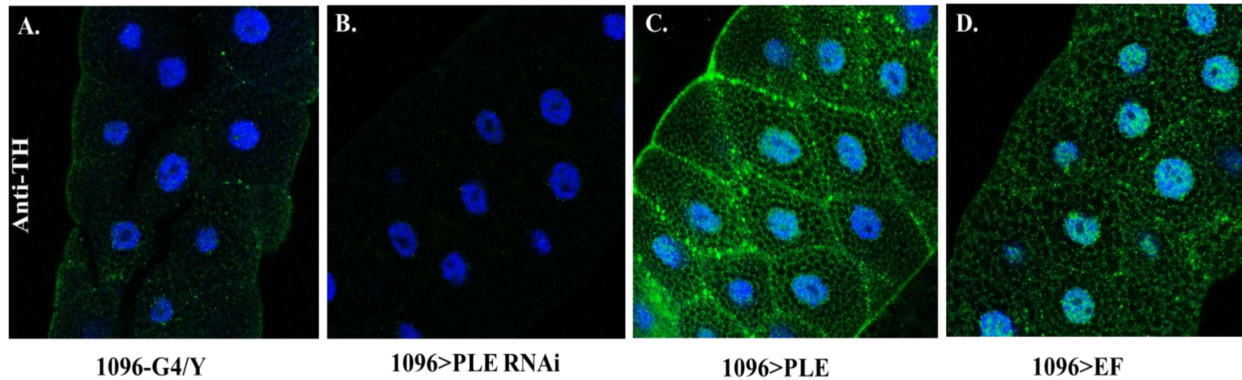

Figure 10: Spatial distribution of Drosophila of Tyrosine Hydroxylase in the Salivary Glands. **(A-D) (A)** Immunohistochemical staining with the anti-DTH antibody recognized signal in the cellular junctions and nuclear periphery, with minimal distribution in the cytosol. **(B)** Complete inhibition of signal when the PLE RNAi is expressed; whereas, the over-expression **(C)** shows a widespread increase in DTH throughout the nucleus, nuclear periphery, cytoplasm, and cellular junctions. **(D)** EF expression lead to an increased expression of DTH in the nucleus and cytosol.

To further evaluate the findings from our fly model system, we idenifited the conservation between PLE in drosophila to homolog of PLE in humans, Tyrosine hydroxylase (TH). Protein sequence analysis revealed 67% homology between DTH/PLE and mammalian TH. Moreover, we found that the phosphorylation site Serine 67 in humans and Serine 32 in *Drosophila* was entirely conserved **(Fig 11)**, adding to the potential overlap between the two model systems.

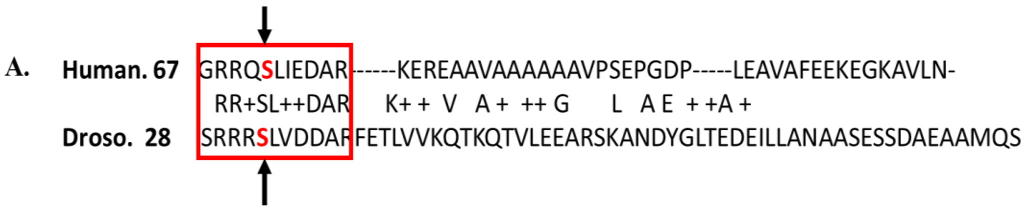

Figure 11: Alignment between Human Tyrosine Hydroxylase and *Drosophila* Tyrosine Hydroxylase. The phosphorylation site of Human TH and DTH are boxed in red, with the serine phosphorylated also red.

It is understood that EF can activate PKA via production of cAMP. In response to this signal, PKA may phosphorylate DTH, potentially leading to an up-regulation of melanin mediated by the increase in activity and expression of DTH (Wittkopp). We tested this hypothetic mechanism in mammalian cells. We started our analysis with the Human Brain Microvascular Endothelial Cell line, HBMEC, which is a well-studied cell line used in previous lab experiments, and is sensitive to the EF toxin.

Cells were either untreated or treated with ET, the bipartite toxin consisting of EF and PA. Immuno-fluorescence staining of TH in the infected HBMEC cell line revealed that there is indeed some interaction between ET and TH. Untreated cells had TH localization only in the cytoplasm **(Fig 12A)**; whereas in ET-treated cells, there was an evident increase in TH expression levels globally throughout the cell **(Fig 12B)**. In addition, the cellular morphology is altered, which is the conventional phenotype observed in ET-treated cells. Probing for active (phosphorylated) TH showed signal predominantly in small clusters within the nucleus, with minimal signal in the cytoplasm **(Fig 12C).** The EF treated cells show a strong increase in Phospho-TH evident

throughout the cell, with large clusters of signals in the nucleus **(Fig 12D),** demonstrating a clear link between ET and activation of TH.

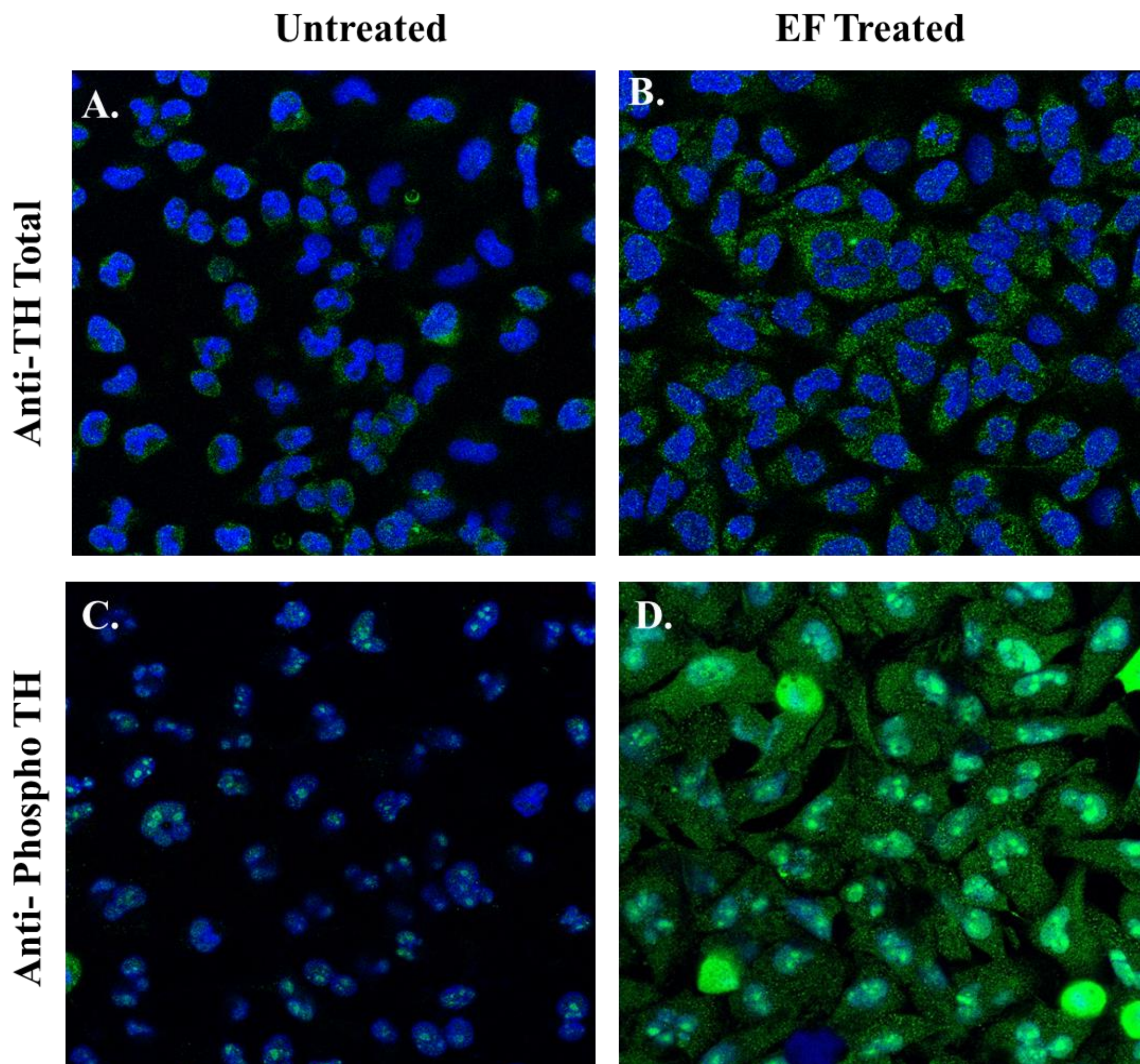

Figure 12: Spatial distribution of Total TH and Active TH in the HBMEC Cell line. **(A-B)** TH distribution in untreated cells was found throughout the cell, while EF treated cells had a significant increase in TH expression globally. **(C-D)** Active TH was found predominantly in the nucleus, with mnimal signal in the cytoplasm, when treated with EF more robust signal appeared in the nucleus and in the cytoplasm.

Collectively, our data suggests that the toxins responsible for Anthrax are also responsible for the intensification of pigmentation, and that particularly EF induces the pigmentation in our fly model system. More specifically, the presence of EF increases the expression levels and activity of DTH, and in-turn elevates pigmentation levels as DTH is the key enzyme responsible for melanogenesis in invertebrates. Moreover, we further tested our model system in a mammalian cell line supporting the interaction between EF and TH is conserved. Therefore, our study has potentially recognized a novel role of the virulence factor, EF, in inducing melanin production causing back eschars during Cutaneous Anthrax.

DISCUSSION

Using transgenic *Drosophila* and human endothelial cells, we have uncovered a link between the EF Adenylate cyclase and Tyrosine Hydroxylase (TH), a key enzyme in melanin synthesis. Production of cAMP by EF leads to activation and increased expression of TH, which elicits pigmentation phenotypes. This relationship needs to be better defined in higher order-organisms and requires further analysis in murine and human cell model systems. Vertebrates all rely on tyrosinase for pigmentation, while *Drosophila* lacks this gene entirely and relies solely on TH to initiate melanin synthesis. In mammals, TH is not essential for pigmentation and is only required for catecholamine synthesis, whereas in *Drosophila*, TH has a dual functional role required for pigmentation and catecholamine synthesis **(Fig 4).** The complete knockout of the TH gene in mice is lethal in development; however, if the precursor molecules necessary for catecholamine synthesis are injected into the embryo, tyrosinase can functionally replace TH and produce the proper components necessary for catecholamine synthesis. Thus tyrosinase and TH are partially interchangeable at the functional level in mammals (Rios).

Although, the only literature on this subject has shown evidence that LF is the main source of pigmentation in melanoma induced mice our data challenges these findings as EF alone drives the increased pigmentation phenotype in *Drosophila*. The earlier report from Koo. Et al. puts great emphasis on LF as the key factor for inducing pigmentation, but in the presence of EF, their synergistic interaction results in elevated melanin production (Koo). However, in this experimental setting, EF independently did not cause an increase. Conversely, our fly data shows that EF triggers melanogenesis

when using the wing and fat body drivers **(Fig 7)**. In contrast, when we expressed LF in the fat body and wing disc, we did not recognize any notable pigmentation phenotype, suggesting that LF is not required for these phenotypes. An explanation for this could stem from the fact that flies simply lack the tyrosinase gene (Wittkopp). Tyrosinase, is regulated by MAPKK and is the essential enzyme for pigmentation in vertebrates and thus LF's ability to inactivate MAPKK will not have any effect on tyrosinase because it is not present in *Drosophila* (Wittkopp). This could explain the lack of contribution of LF to pigmentation phenotypes in flies, in opposition to EF. It is a possible that EF is the key source of pigmentation in our invertebrate system, whereas in vertebrates, EF and LF both synergistically induce pigmentation, with LF playing a more critical role. Conversely, it is worth noting that cAMP analogs, as well as forskolin and cholera toxin, which trigger an artificial rise in cAMP, can independently cause pigmentation in mice and humans (Passeron). Therefore, it is conceivable that EF can have analogous effects.

We have an established fly model system which recapitulates many aspects of anthrax toxins activity, and we are planning to use a melanoma B16 cell line system to investigate Anthrax toxins specific effect on melanogenesis. The B16 cell line is the conventional murine tumor cell line used as a model for experiments in melanocytes, and have been exploited by other groups using drugs that recapitulate the similar cellular responses to EF and LF (Englaro). In conjunction, we plan to infect the B16 cell line with Anthrax, then measure Tyrosinase activity and expression levels, as well as its cellular localization. We also plan to measure melanin levels following infection with different strains of wt B. a, or strains lacking either or both toxins, This would aid in

understanding how the pigmentation synthesis pathway responds to EF and LF, and to help determine whether EF or LF is the main culprit for the pigmentation phenotype. Overall, these findings would challenge or support the previous work from Koo. Et al. Moreover, we believe it is equally important to monitor the expression levels of the additional proteins responsible for melanogenesis such as: Rab27a, Tyrosinase related protein 1, and Tyrosinase related protein 2 to validate our hypothesis that Anthrax triggers melanin synthesis in infected skin regions.

Supportively, a group aimed at understanding melanocyte differentiation and cellular morphology through inhibition of MAPkinase found similar results to our projected melanocyte Anthrax model. The group used forskolin, a cAMP induction analog, and PD98059, a MAPkinase inhibitor, both of which similarly reproduce EF and LF phenotypes respectively (Englaro). Their findings paralleled our hypothesized melanocyte model system, showing an increase in tyrosinase expression and activity as well. Interestingly, they also looked at melanocyte morphology, particularly changes in number and length of dendritic protrusions, which showed an overall increase in dendricity in response to these treatments.

In addition to the B16 model system, we are also interested in generating a murine model system to recapitulate a live Anthrax infection. Vegetative bacteria can be injected into the skin resulting in inflammation and swelling. Currently, similar experiments have been conducted for short periods of time (a few hours) in white mice lacking tyrosinase (Guichard) but could be extended for several days and performed on black mice that are able to produce melanin. Additionally, we could further isolate skin patches to reproduce similar experiments used on cultured cells, such as testing the

levels and activity of TH. This would help ensure that potential in-vitro findings with B16 are validated in an *in-vivo* mouse infection model. Moreover, these findings should be representative of Cutaneous Anthrax because it epitomizes the disease using the actual route of infection rather than transgenic expression of EF and LF used in our *Drosophila* model system. Importantly, the mouse B.a infection system utilizes a live host organism which is essential for a systemic representation of the disease; whereas, the B16 cell line represent only one cell type, isolated from its in vivo biological context. Both findings from these experiments will help identify and support our proposed hypothesis on Anthrax's role in melanogenesis.

Understanding the cellular relationship of Anthrax and melanogenesis is one of the goals of this project, in-addition to this, we also aim at understanding the purpose of the black eschars. We are curious if these eschars are a host defense system, where our body expresses melanin as a by-product to limit the infection of *Bacillus antrhracis*, or the up-regulation of melanin is an additional mechanism the pathogen exploits to promote greater virulence. This question has not been answered by other groups, and should be seriously considered because it gives rise to potentially other therapeutic avenues to further treat Anthrax as well as creating the pipeline of studies to further investigate melanin as an immune defense system towards invading skin pathogens.

It's conventionally known that melanin plays a supportive role as a nuclear barrier protecting the nucleus from UV radiation, and ultimately, preventing DNA damage (Kalamas). Questionably, there are other hypothesized roles of this multi-faceted polymer because melanin is not specific to the epidermis, but actually found in other tissues as well such as: throat, nasal cavity, auditory passage, and the retina

(Sviderskaya). Moreover, regions of the skin that are not exposed to light are often darker than other regions that are such as the genitalia of humans, even seen in new-borns that have never been exposed to light. Nocturnally active species such as bats have dark fur, and even some of the internal membranes of animals are melanin rich, specifically: the epithelium of the inner ear, uveal tract of the eye, peritoneum of fish and amphibians (Zuasti). The role of melanin is these animals and tissues is still uncertain, with minimal research focused on understanding the main function of this ubiquitously found polymer. However, it is clear that UV protection is not its only function.

The process of melanization is highly conserved and the conserved developmental pathways required to produce melanocytes suggest that melanin is ancient and necessary for survival (Robins). Melanocytes are derived from melanoblasts that stem from the neural crest in the first two months of human development, indicating that melanin has a role in different organ systems (Sviderskaya, Robins).

In invertebrates, it is agreed that melanin is used for antimicrobial defense, as various parasites and bacteria trigger melanin production by activating the prophenol-oxidase cascade in the final steps in melanin production. For example, the eukaryotic parasite *Plasmodium* triggers the phenol oxidase system facilitating melanin synthesis. The initial stimulatory mechanism is unclear; however, the Raper-Manson pathway is further utilized to conventionally synthesize melanin. Lipopolysaccharides and peptidoglycan from the pathogen are the major initiating factors that trigger melanization (Chun). The melanin produced forms a coat around in the infectious agent, and then suppresses and kills the microorganism. It is hypothesized that melanin's aromatic

structure is used to interact and degrade the lipid bilayer of the bacterium. In addition, radical by-products are formed in the melanin synthesis process and have shown preliminary evidence that they too exert anti-microbial activity (Riley).

Evidence from previous studies show immunity and melanin synthesis pathway share similarities at the genetic, biochemical, and functional levels. Attractin, a protein found in both skin and in the immune system, is localized on multiple different immune-cell types and accumulates on the surface of T cells. In the epidermis, attractin positively regulates melanogenesis by binding to the agouti protein and inhibiting it's function (Duke-Cohan). In turn, the agouti protein binds to alpha MSH preventing it from binding to the MC1R and inducing melanogenesis. In addition, melanin suppresses the tumor necrosis factor, which is a critical meditator of inflammation and immunity. Moreover, in that same study, melanin in-vivo and in-vitro inhibited the production of inflammatory responses: IL6, IL-Beta, IL10, further illustrating the link between melanin and immunity (Mohagheghpour). The most significant finding supporting this relationship was shown in a genetic study that linked albinism and impaired immunity. The groups examined Hermansky Pudlac Syndrome (HPS) and Griscelli Sydnrome (GS) two genetic dispositions leading to hypopigmentation phenotypes, and found reduced antimicrobial defense in the skin of these patients (Menasche). Ultimately, considerable evidence points out to an existing link between immunity and melanin.

It is essential to also note the prominent role of melanocytes in immunity, as these cells represent the primary source of melanin, but also house the machinery necessary to synthesize melanin. Melanosomes have been defined as a variant of

lysosomes because they possess lysosomal-like features such as proton pumps that help promote an acidic environment within the organelle and lysosomal hydrolases necessary for protein degradation. In an experiment, phagosomes found on the epidermis were tagged with GFP and showed to deliver their content of debris to melanosomes for further degradation (Schraermeyer), signifying a structural and functional overlap. Additionally, melanocytes contain histamine receptors, which are identically found on lysosomes, and actively induce melanogenesis when histamine binds to these receptors emphasizing the link between melanosomes and innate immunity (Yoshida).

Conversely, not all literature points at melanin as an immune defense polymer. The fungal pathogens, *Crpytococcus neoformans* and *Aspergillus fumigatus*, have melanin deposits that coat their surface and have been recently confirmed through mutagenesis studies to exploit these polymers as virulence factors necessary to propagate infectivity and lethality (Liu). Through direct intracerebral injection of C. *neoformans*, the pigmented pathogen induced significant CNS damage with reduced inflammatory cytokine response. Whereas, when the pathogen was defective for melanin synthesis— there was an increase in cytokine mRNA, and the pathogen did not evade the immune response, nor was the infection harmful (Barluzzi). Moreover, A. *fumigatus* commonly evades host immunity and is insusceptible to neutrophil phagocytosis; however, in the absence of melanin the mutant was susceptible to phagocytosis, and ultimately, un-infective (Tsai). In essence, this reinforces the multi-faceted functionality this polymer has, as well as questioning its function in cutaneous anthrax.

Notably, melanin does not only function as a protective shield against UV radiation, but also shares innate immune properties that are necessary for survival of most organisms. Melanocytes and melanosomes are necessary at attenuating the effects of the pathogen, and potentially explains why cutaneous anthrax is far less lethal compared to other routes of infection. Indeed, inhalation and ingestion of anthrax spores is significantly more lethal and has a higher mortality rate (Kalamas), which then strikes the question of whether the melanin present in the skin has a role in reducing the spread of the pathogen. This addresses further avenues to pursue in treating the different modes of Anthrax infection, as well as a potentially novel direction to treat dermal pathogens. One could imagine the induction of melanin could further help prevent skin infections of dermal diseases such as atopic dermatitis, and cellulitis.

We believe we have established an understanding of the black eschars prevalent in cutaneous anthrax individuals, and we further aim at supporting our findings with a mammalian B16 cell-line. We have recognized a potentially novel role of the virulence factor, EF. In addition, we are interested in addressing the questionable role of melanin in this disease. We are curious if its only role is to synthesize these dark patches on the skin, or if there is a deeper innate immune function present in the infection. Again, anthrax dates to antiquity with the name delineating from coal and thus understanding the cellular mechanism of these black eschars as well as melanin's role in the disease is essential for fitting the missing puzzle pieces in understanding this ancient disease.

This thesis, in full, is currently being coauthored with Annabel Guichard, Ph.D. Jammal Abu-khazneh was the primary author of this thesis.

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