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## Deciphering the Role of Glycine134 in the Human Acidic Growth Factor-1's Binding to Heparin

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***Deciphering the Role of Glycine134 in the  
Human Acidic Growth Factor-1's Binding to  
Heparin***

An Honors Thesis submitted in partial fulfillment of  
the requirements of Honors Studies in Biology

By

Adam Wesley Burroughs

2016

Biology

J. William Fulbright College of Arts and Sciences,  
**The University of Arkansas**

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**Acknowledgements:**

This work is supported by NIH(P30 GM 103450), NSF grant IOS-0842937, DOE grant DE-02-01ER15161, the Arkansas Bioscience Institute grants to TKSK, and University of Arkansas Fulbright 2015 Honors College Research Grant. I would like to thank my close friends for working alongside me: Josh Anderson, Ellen Fields, and Roshni Patel. I would also like to give special thanks to Dr. T.K.S. Kumar, Dr. Srinivas Jayanthi, and Julie Eberle for their continual encouragement, advice, and kindness. Last, I would like to thank the University of Arkansas Honors College for supporting my travel to present this work at the American Chemical Society (ACS) Southeast/Southwestern Regional Meeting in Memphis, TN.

**Abstract:**

Human acidic fibroblast growth factor 1 (FGF-1) is a potent modulator of cell survival and exhibits a universal role in various physiological processes. Though potent, FGF-1 unbound to heparin is known to show a poor thermal stability and a relatively short in vivo half-life. Much is known about the structure and relation of FGF-1 with heparin yet there is still unknown information regarding the exact role of heparin in stabilizing FGF-1. Thus, the aim of this study is to mutate glycine at position 134 to glutamic acid in wild type FGF1. G134 is located in the heparin binding pocket, thus the effects of this mutant will provide direct information on the interaction between heparin and the mutant form of FGF1. Because G134 is in close proximity to residues R136 and R133, the incorporation of glutamic acid, a negatively charged residue, in the heparin binding pocket might contribute to the formation of new electrostatic interactions in the binding pocket that would plausibly affect the binding affinity of FGF1 to heparin. FGF-1 G134E has been characterized using various biophysical methods. Results of the mutant characterization will be discussed in greater detail.

**Introduction:**

I. FGF overview: Human fibroblast growth factors (FGFs) are potent modulators of cell survival and are well-known universal ligands participating in various physiological processes (Dubey and Blaber 2005). FGF's ubiquitous nature are manifest in their evolutionary history; with all FGFs sharing conserved mechanisms in both invertebrates and vertebrates (Itoh and Ornitz 2004; Popovici et al. 2005). FGF signaling begins, for most FGF families, with a signal peptide that destines FGF out of the interior of the cell and into the extracellular matrix (ECM). However, FGF1, this paper's protein of interest, is not secreted through the endoplasmic reticulum—Golgi secretory pathway with a signal peptide; rather, FGF1 is secreted independently into the ECM (Nickel 2005 and Revest et al. 2000). Once in the ECM, the fate of FGF varies depending on the FGF family: FGF may be relocated by a carrier protein, or relocation may occur through the digestion of the ECM. Once relocated, FGFs have the ability to directly affect target cells (Powers et al. 2000). In the ECM, FGFs bind with high affinity to heparan sulfate proteoglycans (HSPG) where FGF may carry out cell communication by either autocrine or paracrine signaling (Suh et al. 2014). Binding of FGF1 to HSPG is crucial, because a HSPG is required for the tyrosine kinase, FGF receptor (FGFR), to recognize FGF1 as a signaling peptide (Pineda-Lucena et al. 1996; Stauber et al. 2000; Ornitz and Itoh, 2001; Eswarakumar et al. 2005). Once the FGF1-HSPG complex binds to the FGFR on the cell surface, FGF1 may deliver its mitogenic effects (Johnson and Williams 1993; Schlessinger 2000). FGF1's mitogenic activity is mediated by a conformational change in the FGFR, inducing receptor dimerization, ultimately leading to the "beacon" of

signaling events characteristic of tyrosine kinase receptors (Powers et al. 2000, Ornitz et al. 1992, Schlessinger et al. 2000 and Yayon et al. 1991).

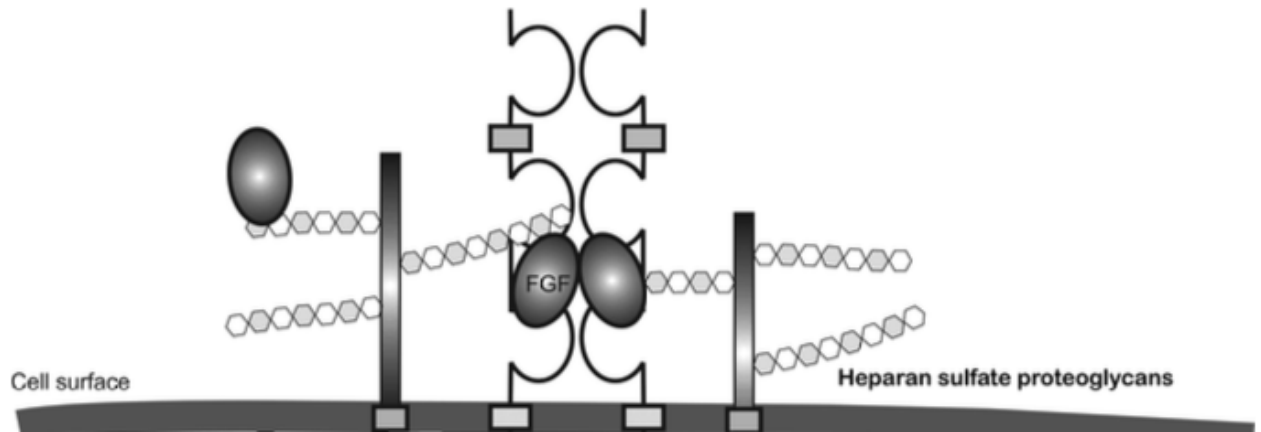


Figure 1: Cartoon depicting that heparan sulfate proteoglycans (HSPGs) are intimately associated with FGF receptor binding on the cell surface (Zakrzewska et al 2008).

II. FGFR overview: The FGF receptor is encoded by four genes, *FGFR1–FGFR4*, which contain the blueprints for the receptor’s structure: three extracellular immunoglobulin (Ig) like domains (D1-D3), one single-pass membrane spanning domain, and the cytoplasmic tyrosine kinase domain (Mohammadi et al. 2005). A defining feature of the FGFR is called the “acid box”, a serine-rich amino acid sequence in the linker region between the extracellular D1 and D2 domains (Beenken and Mohammadi 2009). The acid box and the D1 domain are thought to play a role in receptor autoinhibition (Wang et al. 1995). In addition, the extracellular D2-D3 domains are responsible for the FGF1-HSPG complex binding and specificity (Beenken and Mohammadi 2009).

There are multiple combinations of FGF and FGFR, as most FGFs can bind to and activate several receptors; and even further permutations are possible due to several isoforms of the FGFR being in existence (Zakrzewska et al. 2008; Beenken and Mohammadi 2009). Two isoforms of the FGFR, b and c, are formed by alternate splicing of the D3 domain of the FGFR 1-3 transcript. These isoforms have different FGF binding specificities, with the b and c isoforms being epithelial and mesenchymal, respectively (Johnson et al. 1991). Interestingly, all FGF family members bind to either epithelial or mesenchymal FGFRs but FGF1 has the ability to activate both splice isoforms of the FGFR (Beenken and Mohammadi 2009).

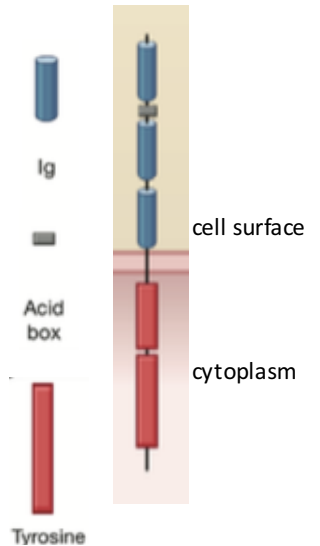


Figure 2: Cartoon depicting the FGF receptor (FGFR) which consists of: three extracellular immunoglobulin (Ig) like domains (D1-D3), the acid box, one single-pass membrane spanning domain, and the cytoplasmic tyrosine kinase domain (Lemmon and Schlessinger, 2010).

### III. Ligand-receptor interaction and the triggering of downstream events:

A. Receptor Dimerization: After the binding of the FGF1-HSPG complex to the FGFR, autophosphorylation of the FGFR occurs. Looking at the big picture, autophosphorylation of the tyrosines, in the cytoplasmic tyrosine kinase domain, marks a major event for FGF1 signaling. There is a boost in activity of the receptor kinase, and the recruitment and activation of downstream signaling molecules as they bind to the phosphotyrosines of the FGFR (Eswarakumar et al. 2005). Importantly, autophosphorylation of the receptor serves as an “on-switch” for many broad reaching cellular processes like that of proliferation, differentiation, and migration (Zakrzewska et al. 2008).

B. Receptor Activation: Specifically, autophosphorylation occurs when the FGFR dimerizes allowing the cytoplasmic kinases domains to “crossphosphorylate”, or

transphosphorylate, on A loop tyrosines which become activated. Phosphorylation of residues on A loop is followed with phosphorylation of the tyrosines in the C tail, which are kinase insert and juxtamembrane regions (Mohammadi et al. 1996). A phosphorylation at tyrosine 766 (Y766) in the C tail on the FGFR is necessary for the binding and activation of one of the main intracellular downstream signaling molecules—phospholipase C (PLC)  $\gamma$ . Phosphorylated Y766 creates a binding site for the interaction of PLC $\gamma$ 's SH2 domain (Beenken and Mohammadi 2009). After the activation of PLC $\gamma$ , downstream messengers like IP3 and DAG are activated leading to an increase in cytoplasmic calcium levels and activation of protein kinase C (PKC).

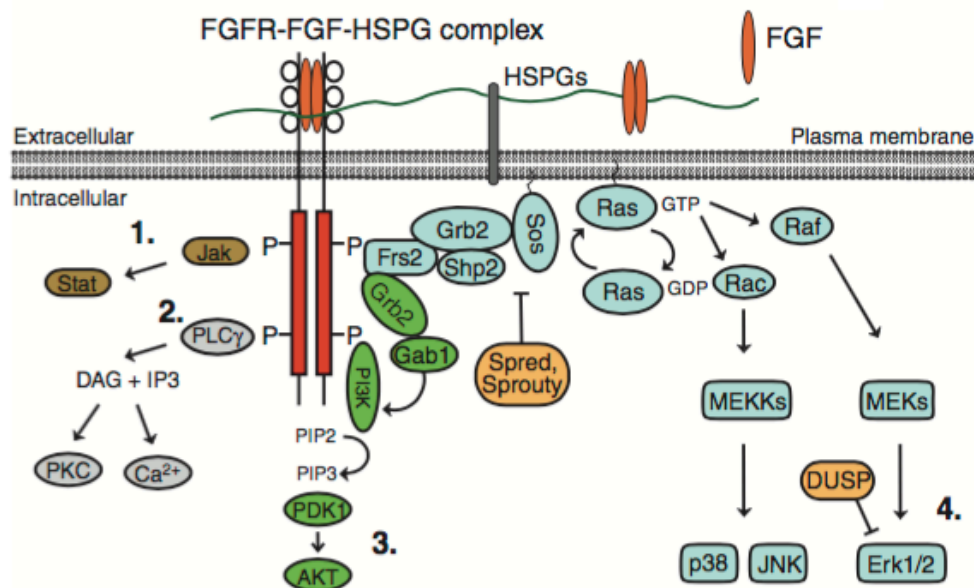


Figure 3: Cartoon showing the four main signaling pathways triggered by the interaction of FGF-FGFR (labeled 1-4): 1, Janus kinase/signal transducer and activator of transcription; 2, phosphoinositide phospholipase C, 3, phosphatidylinositol 3-kinase; and 4, mitogen-activated protein kinase (Lanner and Rossant 2010).

Another important downstream substrate of the FGFR is FGFR substrate 2 (FRS2). In contrast to PLC $\gamma$ , FRS2 does not bind to the phosphotyrosine domain; rather FRS2 binds to the juxtamembrane region of the FGFR. The constitutive association of

FRS2 to the FGFR receptor leads to FRS2 phosphorylation, triggering the activation of the Ras and PI3 kinase–Akt signaling pathways (Dailey et al. 2005).

C. Receptor endocytosis: Signaling from the FGFR is not limited only to the membrane, but the FGFR also exhibits activity in the cytosol and nucleus of cells. This activity of the FGFR out of the cell membrane is made possible through the endocytosis of the activated FGF–FGFR complexes and also through sources of ligand within the cell (Wiedlocha and Sorensen 2004). The FGF–FGFR complexes are endocytosed by the clathrin-mediated pathway; however, other mechanisms for the internalization of FGF–FGFR have been postulated (Marchese et al. 1998, Citores et al. 1999, and Citores et al. 2001).

IV. FGF’s interaction with heparin: Though FGF1 is potent, apart from HSPG, FGF1 is known for having poor thermal stability and a relatively short in vivo half-life (Culajay et al. 2000). The 154 amino acids of FGF1 are oriented so that regions important for folding are distributed within the protein with regions associated with the function i.e., heparin binding affinity and receptor binding affinity that fold late in the folding pathway (Longo et al. 2012). Most known members of the FGF family contain a HSPG binding pocket, where the binding of FGF1 to heparan sulfate proteoglycans protects FGF1—shielding it against proteases and thermal denaturation—in addition to aiding FGF1 to bind to and activate its FGFR. (Rapraeger et al. 1991 & Yayon et al. 1991). The structure of HSPG can be simplified as decasaccharides consisting of a helix arranged by repeating  $\alpha$ -1-4 linkage disaccharide units of D-glucos-amine and L-iduronic acid (Schlessinger et al. 2000). HSPGs are referred to as “sugar” proteins (glycoproteins) all of which contain one or more covalently attached heparan sulfate chain and a core protein

(Esko et al. 2009). The core protein can either traverse the cell membrane or it can be anchored to the membrane by a glycosylphosphatidylinositol (Zakrzewska et al. 2008). Heparin is a highly sulfated form of heparan sulfate, therefore negatively charged, and is found at the cell surface and in the extracellular matrix where it interacts with FGF1 and numerous other ligands (Sarrazin et al. 2011). HSPGs act as a template to bring together the FGF1 ligand and receptor by simultaneous binding both FGF and FGFR (Sarrazin et al. 2011). This synchronous binding helps stabilize the interactions between FGF1 and its FGFR (Hacker et al. 2005). Also noteworthy, HSPGs enable FGF to bind to several FGFRs (Zakrzewska et al. 2008). In contrast to the FGFR, HSPGs are in greater abundance and form oligomeric complexes with FGF1 (Zakrzewska et al. 2008). Due to FGF1's need to interact with heparin for FGFR activation, FGF1 displays what is called a “dual-receptor model” of binding. Though FGF1 interacts with relative high affinity to HSPGs, the dual receptor model depends on FGF interacting with both a “low affinity receptor”, HSPG, and “high affinity receptor”, FGFR (Rapraeger et al. 1991, Yayon et al. 1991, Spivak-Kroizman et al. 1994, Klagsbrun et al. 1991, and Ornitz et al. 1992).

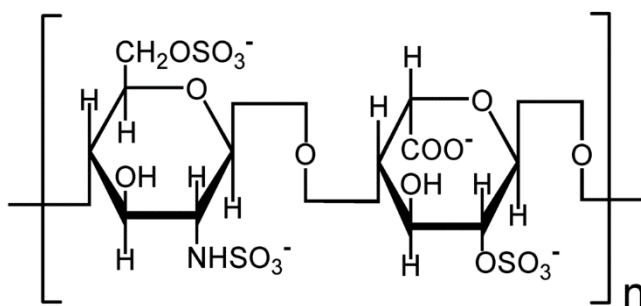
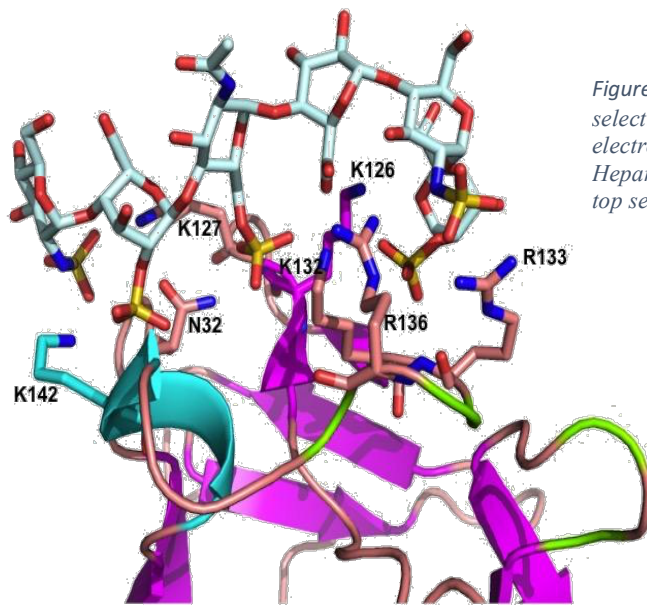


Figure 4: Image showing the chemical structure of the repeating (glucosamine — L-iduronic acid) units of heparin. (Ou et al. 2015)

The HSPG binding site is located inside the core of FGF which is made up of the  $\beta 1$ – $\beta 2$  loop and parts of the region across  $\beta 10$  and  $\beta 12$  (Beenken & Mohammadi 2009).

Because FGF1 binds to heparin with high affinity, immobilized heparin affinity chromatographic resins are used for purification of FGF1 and many other growth factors. As mentioned above, HSPGs are required if FGF1 is to activate the FGFR and induce its signaling cascade. Therefore, HSPG is characterized as a “coreceptor” because its function is to bind to FGF1 (and other ligands) to form complexes that may trigger a conformational change of the ligand and/or the receptor. Because heparin is sulfated it forms electrostatic interactions with FGF. Particularly, some of the positive residues of FGF1 that interact with heparin are K126, K127, R133 and R136 to name a few.



*Figure 5: PyMOL image showing heparin and FGF-1 bound with a selection of some labeled amino acid residues that participate in the electrostatic interactions with heparin in the FGF1 binding pocket. Heparin is represented by the primarily light blue stick model in the top section of the cartoon.*

V. FGF Family member’s overview: To date, there are 18 known mammalian FGFs classified into 6 subfamilies (1, 4, 7, 8, 9, and 19) based on sequence homology and evolutionary history (Itoh and Ornitz, D 2004). FGF1 and FGF2 are grouped into the FGF1 subfamily; FGF4, FGF5, and FGF6 are grouped into the FGF4 subfamily; FGF3,

FGF7, FGF10 and FGF22 are grouped into the FGF7 subfamily; FGF8, FGF17, and FGF18 are grouped into the FGF8 subfamily; FGF9, FGF16, and FGF20 are grouped into the FGF9 subfamily, and FGF19, FGF21, and FGF23 are grouped into the FGF19 subfamily.

There is a seventh FGF subfamily, the FGF11 subfamily, consisting of FGF members FGF11-FGF14. However, it was discovered that the FGF11 subfamily do not activate FGFRs, even though they display high sequence homology with the FGF family (Olsen et al. 2003). Therefore, the FGF11 subfamily members are generally no longer considered members of the FGF family (Olsen et al. 2003).

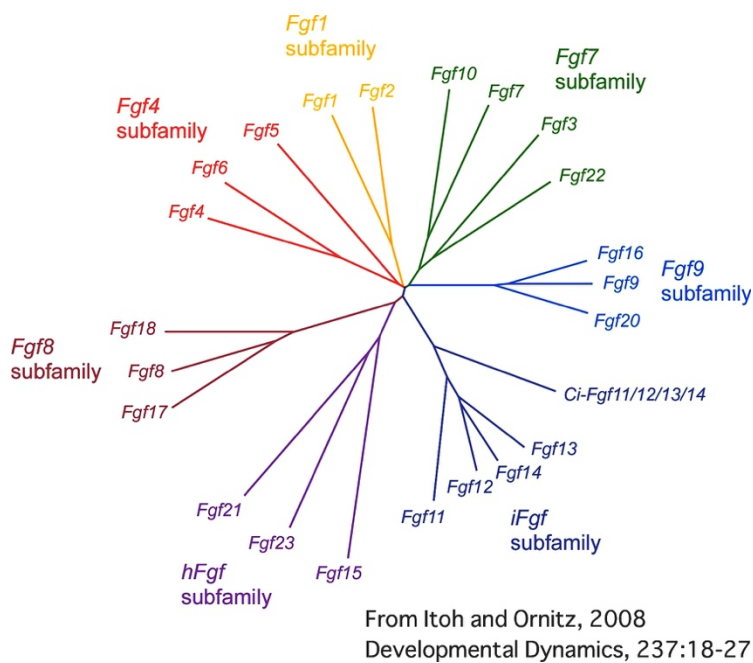


Figure 6: Evolutionary relationships between the twenty-two identified FGFs in mice. The “Fgf subfamily” is also referred to as the FGF 11 subfamily, which do not activate FGF receptors. The “hFgf subfamily” is sometimes referred to as the FGF19 subfamily. Note: FGF15 represents the mouse ortholog of human FGF19 (Itoh and Ornitz 2008).

All FGF family members share a high affinity for heparin and heparan-like glycosaminoglycans (Burgess and Maciag 1989). In addition, all FGFs also share a homologous central core of 120-130 amino acids arranged into 12 antiparallel  $\beta$ -strands ( $\beta$ 1- $\beta$ 12) with different amino and carboxyl terminals (Beenken and Mohammadi

2009). This difference in amino and carboxyl tails is what accounts for the different biological effects of the FGF family members (Mohammadi et al. 2005). The acronym of FGF, “fibroblast growth factor”, may be misleading in implying that each FGF stimulates fibroblasts; this is not the case—FGF7 does not stimulate fibroblasts (Powers et al. 2000). Rather, the FGF initials are meant to show that each factor belongs to the same family, because they all share common structure—with the exception of FGF11-FGF14 not being regarded as FGF family members, as mentioned above.

FGFs regulate an eclectic group of developmental processes. The first five subfamilies of FGF, which includes FGF1, are paracrine factors and have roles in tissue patterning and the creation of organs in development (Beenken and Mohammadi 2009). In adults, members of the FGF19 subfamily have been shown to act in an endocrine manner by regulating cholesterol, bile acid, glucose, vitamin D, and phosphate homeostasis (Fu et al 2004, Kharitonov et al. 2005, Razzaque and Lanske 2007, and Tomlinson et al. 2002). The following contains tables, addressing each mammalian FGF subfamily and their individual members.

FGF Ligand:	Molecular Weight in kDa:	Functional Activity:	Refs:
FGF1, this paper's protein of interest.	16 <sup>8</sup>	<u>Physiological Role:</u> The physiological role of FGF1 is remains uncertain, although it is thought to play some part in the maintenance of vascular tone <sup>1,2*</sup> . <u>FGF1 Knockout:</u> Interestingly, FGF1 knockout mice exhibit no apparent complications <sup>3</sup> . <u>Medical Applications:</u> FGF1 has been shown to: regenerate transected spinal cords in rats <sup>4</sup> , improve collateral artery growth and capillary proliferation <sup>5</sup> , treat insulin resistance and type 2 diabetes in mice <sup>6</sup> , and in combination with nerve grafts, FGF1 has partly restored movement to the paraplegic <sup>7</sup> .	<sup>1</sup> Cuevas, et al. 1991, <sup>2</sup> Cueva et 1996, <sup>3</sup> Miller et al. 2000, <sup>4</sup> Cheng et al. 1996, <sup>5</sup> Schumacher et al. 1998, <sup>6</sup> Suh et al. 2014, <sup>7</sup> Cheng et al. 2004, and <sup>8</sup> Blaber 1996.
FGF2	18 <sup>8</sup> 22.5 23.1 24.2	<u>Physiological Role:</u> Like FGF1, the physiological role of FGF2 is still unsure; in addition, FGF2 is thought to play some role in the maintenance of vascular tone <sup>1,2*</sup> . <u>FGF2 Knockout:</u> like FGF1, FGF2 knockout mice exhibit no apparent complications <sup>3</sup> . <u>Medical Applications:</u> FGF2 is the most studied FGF, where it has therapeutic potential for cardiovascular disease and prostate and renal cancer <sup>4,5,6,7</sup> .	<sup>1</sup> Cuevas, et al. 1991, <sup>2</sup> Cueva et 1996, <sup>3</sup> Miller et al. 2000, <sup>4</sup> Unger et al. 2000, <sup>5</sup> Laham et al. 1999, <sup>6</sup> Simons et al. 2002, and, <sup>7</sup> Sellke et al. 1998, and <sup>8</sup> Florkiewicz and Sommer 1989.

Table 1: The FGF1 subfamily — Molecular weights and general functional activity,

FGF Ligand:	Molecular Weight in kDa:	Functional Activity:	Refs:
FGF4	22 <sup>5</sup>	<u>Physiological Role:</u> FGF4 functions in the formation heart valve leaflets <sup>1</sup> and the development of limbs <sup>2</sup> . <u>FGF4 Knockout:</u> FGF4 knockout mice are lethal due to FGF4's involvement in trophoblast proliferation <sup>3</sup> . <u>Medical Applications:</u> FGF4 has been administered as means of gene therapy for stable angina in women <sup>4</sup> .	<sup>1</sup> Sugi et al. 2003, <sup>2</sup> Sur et al. 2002, <sup>3</sup> Feldman et al. 1995, <sup>4</sup> Henry et al. 2007, and <sup>5</sup> Delli Bovi <i>et al.</i> 1987.
FGF5	32-38 <sup>2,3,4</sup>	<u>Physiological Role:</u> FGF5 serves as a negative regulator of a step in the hair follicle growth cycle <sup>1</sup> . <u>FGF5 Knockout:</u> FGF5 knockout mice have very long hair, without any other complications <sup>1</sup> . <u>Medical Applications:</u> There is a potential to create FGF5 inhibitors to support hair growth <sup>1</sup> .	<sup>1</sup> Hebert et al. 1994, <sup>2</sup> Goldfarb, M. <i>et al.</i> 1991, <sup>3</sup> Zhan <i>et al.</i> 1988, and <sup>4</sup> Bates <i>et al.</i> 1991.
FGF6	25 <sup>4</sup>	<u>Physiological Role:</u> FGF6 plays a role in the development of muscle tissue <sup>1</sup> . <u>FGF6 Knockout:</u> FGF6 knockout mice displayed damaged muscle regeneration where they showed substantial thickening and scarring of connective tissue after a freeze-crush injury <sup>2</sup> . <u>Medical Applications:</u> As of now, there are no current therapeutic applications of FGF6 <sup>3</sup> .	<sup>1</sup> Armand et al. 2006, <sup>2</sup> Floss et al. 1997, <sup>3</sup> Beenken and Mohammadi 2009, and <sup>4</sup> Coulier <i>et al.</i> 1991.

Table 2: The FGF4 subfamily — Molecular weights and general functional activity.

FGF Ligand:	Molecular Weight in kDa:	Functional Activity:	Refs:
FGF3	28-32 <sup>3,4</sup>	<u>Physiological Role:</u> FGF3 serves a very specific role for inner ear development <sup>1</sup> . <u>FGF3 Knockout:</u> FGF3 human knockouts showed deafness in addition to a few dental defects <sup>1</sup> . <u>Medical Applications:</u> As of now, there are no current therapeutic applications of FGF3 <sup>2</sup> .	<sup>1</sup> Tekin et al. 2007, <sup>2</sup> Beenken and Mohammadi 2009, <sup>3</sup> Brookes <i>et al.</i> 1989, and <sup>4</sup> Antoine <i>et al.</i> 1997.
FGF7	28 <sup>7,8</sup>	<u>Physiological Role:</u> FGF7 serves aids morphogenesis <sup>1</sup> . FGF7 is expressed specifically in mesenchyme tissue, where FGF7 concentrations are increased dramatically (150-fold) in skin after skin injury <sup>2</sup> . In addition, FGF7 levels increase after bladder and kidney injury <sup>3,4</sup> . <u>FGF7 Knockout:</u> FGF7 knockout mice show minor abnormalities like matted hair <sup>5</sup> , and have 30% fewer nephrons compared to control mice <sup>1</sup> . <u>Medical Applications:</u> FGF7 is used as Palifermin, an FDA approved, N-terminally truncated form of FGF7 with increased stability. Palifermin is used for the treatment of chemoradiation-induced mucositis in patients undergoing bone marrow transplantation <sup>6</sup> .	<sup>1</sup> Qiao et al. 1999, <sup>2</sup> Werner et al. 1992, <sup>3</sup> Baskin et al. 1997. <sup>4</sup> Ichimura et al. 1996, <sup>5</sup> Guo et al. 1996, <sup>6</sup> Spielberger et al. 2004, <sup>7</sup> Rubin <i>et al.</i> 1989, <sup>8</sup> Werner 1998
FGF10	30 <sup>6</sup>	<u>Physiological Role:</u> FGF10 serves in branching morphogenesis <sup>1</sup> . It is a presynaptic organizer that serves in vesicle clustering and neurite branching <sup>2</sup> . <u>FGF10 Knockout:</u> FGF10 knockout mice do not develop limbs and lung structures <sup>1</sup> . <u>Medical Applications:</u> FGF10 and FGF7 are thought to play a part in the pathogenesis of prostate cancer by promoting epithelial cell proliferation <sup>3,4</sup> . However, there are no current therapeutic applications of FGF10 <sup>5</sup> .	<sup>1</sup> Kato and Sekine 1999, <sup>2</sup> Umemori et al. 2004, <sup>3</sup> Thomson and Cunha 1999, <sup>4</sup> Yan et al. 1992, <sup>5</sup> Beenken and Mohammadi 2009, and <sup>6</sup> Beer et al. 1997.
FGF22	..	<u>Physiological Role:</u> FGF22 is a presynaptic organizer that serves in vesicle clustering and neurite branching <sup>1</sup> . <u>FGF22 Knockout:</u> FGF22 currently has no knock mice model <sup>2</sup> . <u>Medical Applications:</u> There are no current therapeutic applications of FGF10 <sup>2</sup> .	<sup>1</sup> Umemori et al. 2004, <sup>2</sup> Beenken and Mohammadi 2009

Table 3: The FGF7 subfamily — Molecular weights and general functional activity.

FGF Ligand:	Molecular Weight in kDa:	Functional Activity:	Refs:
FGF8	28-32 <sup>5,6</sup>	<u>Physiological Role:</u> FGF8 plays a role in brain, limb, ear, and eye development <sup>1</sup> . <u>FGF8 Knockout:</u> FGF8 knockout mice are not able to undergo gastrulation <sup>2</sup> and is therefore lethal. <u>Medical Applications:</u> Loss-of-function mutations result in an altered FGFR1c binding or cause Kallmann's syndrome <sup>3</sup> . However, there are no current therapeutic applications of FGF8 <sup>4</sup> .	<sup>1</sup> O'Leary et al. 2007, <sup>2</sup> Meyers et al. 1998, <sup>3</sup> Falardeau et al. 2008 <sup>4</sup> Beenken and Mohammadi 2009, <sup>5</sup> MacArthur et al. 1995, and <sup>6</sup> Gemel et al. 1996.
FGF17	25 <sup>3,4</sup>	<u>Physiological Role:</u> FGF17 is involved in cerebral and cerebellar development <sup>1</sup> . <u>FGF17 Knockout:</u> FGF17 knockout mice show abnormalities in the cerebrum and cerebellum <sup>1</sup> . <u>Medical Applications:</u> As of now, there are no current therapeutic applications of FGF17 <sup>2</sup> .	<sup>1</sup> Xu et al. 2000, <sup>2</sup> Beenken and Mohammadi 2009, <sup>3</sup> Xu et al. 1999, and <sup>4</sup> Hoshikawa et al. 1998.
FGF18	31 <sup>3,4</sup>	<u>Physiological Role:</u> FGF18 is involved in bone development <sup>1,2</sup> . <u>FGF18 Knockout:</u> FGF18 knockout mice express less osteogenic markers and have delayed long-bone ossification <sup>1,2</sup> . <u>Medical Applications:</u> Recombinant FGF18 has anabolic abilities on cartilage <sup>3,4</sup> .	<sup>1</sup> Liu et al. 2002, <sup>2</sup> Ohbayashi et al. 2002, <sup>3</sup> Ellsworth et al. 2002, <sup>4</sup> Moore et al. 2005.

Table 4: The FGF8 subfamily — Molecular weights and general functional activity.

FGF Ligand:	Molecular Weight in kDa:	Functional Activity:	Refs:
FGF9	30 <sup>4,5</sup>	<u>Physiological Role:</u> FGF9 functions in utero in gonadal development and organogenesis <sup>1,2</sup> . <u>FGF9 Knockout:</u> FGF9 knockout mice display male-to-female sex reversal in addition to lung hypoplasia causing postnatal death <sup>1</sup> . <u>Medical Applications:</u> There are currently no therapeutic applications for FGF9 <sup>3</sup> .	<sup>1</sup> Colvin, Green, and et al. 2001, <sup>2</sup> Colvin, White, and et al. 2001 <sup>3</sup> Beenken and Mohammadi 2009, <sup>4</sup> Miyakawa et al. 1999 and <sup>5</sup> Naruo et al. 1993
FGF16	26 <sup>3,4</sup>	<u>Physiological Role:</u> FGF16 plays a role in heart development <sup>1</sup> . <u>FGF16 Knockout:</u> FGF16 knockout mice have substantial cardiac defects and are embryonic lethal <sup>1</sup> . <u>Medical Applications:</u> There are currently no therapeutic applications for FGF16 <sup>2</sup> .	<sup>1</sup> Lu et al. 2008, <sup>2</sup> Beenken and Mohammadi 2009, <sup>3</sup> Konishi et al. 2000, and <sup>4</sup> Miyake et al. 1998.
FGF20	23 <sup>3</sup>	<u>Physiological Role:</u> FGF20 is a neurotrophic factor <sup>1</sup> . <u>FGF20 Knockout:</u> There is currently no FGF20 knockout model <sup>2</sup> . <u>Medical Applications:</u> Currently, FGF20 is being explored for applications in Parkinson's disease <sup>1</sup> .	<sup>1</sup> Ohmachi et al. 2003, <sup>2</sup> Beenken and Mohammadi 2009, and <sup>3</sup> Kirikoshi et al. 2000.

Table 5: The FGF9 subfamily — Molecular weights and general functional activity.

FGF Ligand:	Functional Activity:	Refs:
<b>FGF19</b>	<u>Physiological Role:</u> FGF19, an endocrine FGF, is involved in bile acid homeostasis, lipolysis, and gall bladder filling <sup>1,2,3,4,5,6</sup> . <u>FGF19 Knockout:</u> Display an increased bile acid pool <sup>7</sup> . <u>Medical Applications:</u> There is the potential for recombinant FGF19 to be used in treating diabetes <sup>1,2,4,5</sup> .	<sup>1</sup> Fu et al. 2004, <sup>2</sup> Tomlinson et al. 2002, <sup>3</sup> Xie et al. 1999, <sup>4</sup> Holt et al. 2003, <sup>5</sup> Lundasen et al. 2006, <sup>6</sup> Choi et al. 2006, <sup>7</sup> Inagaki et al. 2005
<b>FGF21</b>	<u>Physiological Role:</u> FGF21, an endocrine FGF, is involved in the fasting response, glucose homeostasis, lipolysis, and lipogenesis <sup>1,2,3</sup> . <u>FGF21 Knockout:</u> There is currently no FGF21 knockout model <sup>3</sup> . <u>Medical Applications:</u> Like FGF19, recombinant FGF21 has the potential for use in diabetes <sup>1,2,3</sup> .	<sup>1</sup> Kharitonov et al. 2005, <sup>2</sup> Nishimura et al. 2000, <sup>3</sup> Beenken and Mohammadi 2009
<b>FGF23</b>	<u>Physiological Role:</u> FGF23, and endocrine FGF, is involved in phosphate homeostasis and vitamin D homeostasis <sup>1,2,3,4,5</sup> . <u>FGF23 Knockout:</u> FGF23 knockout mice display hyperphosphataemia, hypoglycaemia, and Immature sexual organs <sup>2,6</sup> . <u>Medical Applications:</u> Currently anti-FGF23 antibodies are being used in hypophosphataemia <sup>4,5</sup> .	<sup>1</sup> Yamashita et al. 2000, <sup>2</sup> Sitara et al. 2004, <sup>3</sup> Beenken and Mohammadi 2009, <sup>4</sup> Aono et al. 2003, <sup>5</sup> Yamazaki et al. 2008, <sup>6</sup> Shimada et al. 2004

Table 6: The FGF19 subfamily— Molecular weights and general functional activity..

VI. Significance and Design of the G134E Mutation: FGF1, this paper's protein of interest, is a 16-kDa, made of 12 antiparallel  $\beta$ -strands forming a  $\beta$ -barrel – lacking in disulfide bonds (Blaber 1996; Zhu et al. 1991; Stauber 2000; Chi et al. 2001; Ornitz and Itoh 2001). FGF1 possesses incredible therapeutic potential (see table 1 for some examples). The role of FGF1 in human pathology is well studied—where deviant signaling of FGF1 is involved in the pathogenesis of cancer (Ornitz and Itoh 2001; Stauber et al. 2000). In addition, elevated levels of FGF in cancer cells have been shown to be a culprit for the ability of cancer cells to evade chemotherapy treatment (Song et al. 2000). Even more, FGF-1 is already being utilized to promote wound repair and angiogenesis (Thompson et al. 1994; Beenken and Mohammadi 2009). Because of FGF1's broad therapeutic potential, the use of recombinant proteins like FGF is a promising therapeutic approach (Zakrzewska 2008). Previous point mutations of FGF1

have shown to increase thermal stability, reduce proteolytic degradation, prolong and increase mitogenic properties, and increase FGF1's half-life (Zakrzewska 2008).

In this study, a single point mutation was introduced at the 134<sup>th</sup> amino acid position of glycine (Gly/G) to alter the glycine residue into glutamic acid (Glu/E). The overall aim of this group is to create an FGF1 that can be incorporated into wound healing cosmetics. This FGF1 must have increased resistance to proteolytic degradation, and an increased half-life to survive not only in the cosmetic agent but also when it is introduced in vivo (Zorrilla et al. 2010). The engineered mutant of this paper, G134E, incorporated glutamic acid, an amino acid that is known to be polar and negatively charged at physiological pH.

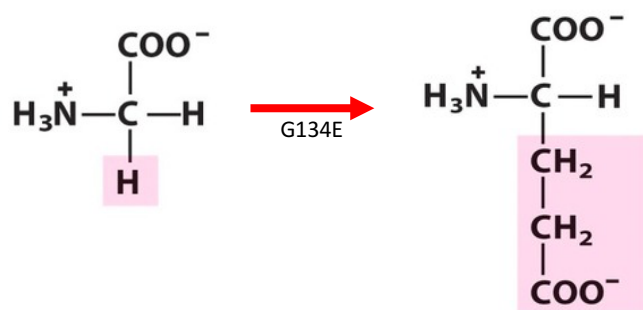
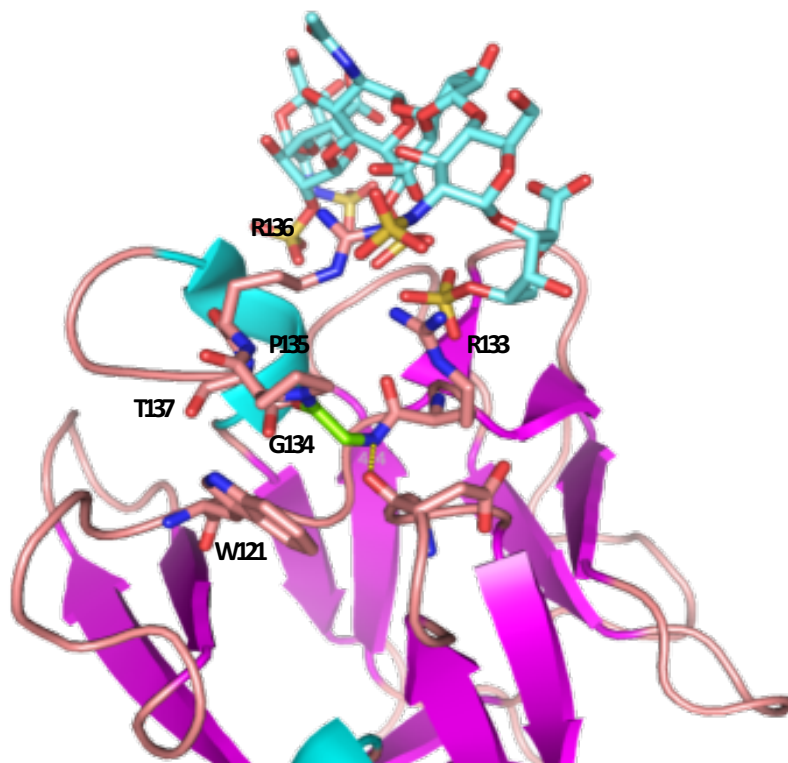


Figure 7: Chemical structures of glycine (left) and glutamic acid (right). The mutant FGF1, G134E, incorporated glutamic acid at the 134<sup>th</sup> amino acid position, replacing glycine.

We hypothesize that incorporation of glutamic acid for the nonpolar and uncharged glycine residue will disrupt the electrostatic interactions and cause a decrease in G134E-FGF1's affinity for heparin. With a newly incorporated long and negatively charged side chain containing carboxyl group protruding from the protein backbone, the negatively charged heparin molecule might be repelled from G134E-FGF1. Proteolytic

enzymes, like thrombin, are known to cleave FGF1 when bound to heparin so discouraging FGF1 to heparin may ultimately achieve the goal of increased proteolytic stability. We also predict that the thermodynamic stability of FGF1 probably would decrease by the neutralization of positive charged amino acid residues in the vicinity. Though the thermodynamic stability may decrease with the G134E point mutation, the incorporation of other point mutations combined with this mutant may discourage heparin binding and increase thermodynamic stability. Expression of the G134E mutant in addition to biophysical characterization experiments were completed to explore the effects of this mutation.



*Figure 8: Microenvironment of residues in the heparin binding pocket proximal to the mutation site of G134.*

## **Experimental Methods:**

The equipment and materials used for this study were of high-quality laboratory grade. For all prepared solutions, double distilled water (ddH<sub>2</sub>O) was used. All assays were performed with protein in an environment with 7.2 pH and 10mM phosphate buffer (PB) with a specified amount of sodium chloride (NaCl). In samples that included heparin, 10x the amount of heparin was used for the given protein concentration unless otherwise specified. For all sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analyses reagents were purchased from VWR. All trichloroacetic acid preparations (TCA) were performed according to the protocol on protein samples before running SDS-PAGE analysis.

### *Bacterial Transformation:*

A plasmid containing the G134E mutation was created by site-directed mutagenesis before the initiation of this study. 1μL of the G134E plasmid was inoculated in 50μL of BL21 (DE3) super competent *E. coli* cells then was set on ice for 30 minutes. BL21 (DE3) super competent *E. coli* cells was this lab's cell-line choice due to their lack of proteolytic enzymes that would degrade G134E and also a show a high efficiency of transformation. The BL21 (DE3) super competent *E. coli* cells containing the plasmid were placed in a 42°C water bath for 45 seconds to uptake the plasmid and then were immediately placed on ice for 3 minutes. After the bacterial cells' uptake of the G134E plasmid, 800μL of sterile lysogeny broth (LB), a nutrient rich medium, was added to the cells. This solution was then incubated in a shaker at 250rpm for 45 minutes at 37°C. A 100μL sample of bacterial cells containing plasmid were uniformly spread on an agar

plate containing the antibiotic ampicillin (Amp). Ampicillin was selected because the plasmid utilized in this study has genes that confer the resistance to Amp; consequently, only the BL21 (DE3) super competent *E. coli* cells containing plasmid were able to survive. The agar plate containing the antibiotic ampicillin (Amp) and bacterial cells were stored upside-down in a 37°C incubator for 14 hours.

#### *Overexpression of G134E-FGF1:*

Small scale expression was completed and checked by SDS-PAGE to verify the G134E-FGF1 mutant was present. Then glycerol stocks of G134E were prepared in the Kumar lab prior to this study and stored in a -80°C freezer. A 1mL glycerol stock was then taken from the -80°C freezer and was added to 150 mL of sterile autoclaved LB media which was allowed to reach room temperature; in addition, 150µL of Amp was added to the solution. The solution was incubated in a 37°C shaker at 180rpm for 14 hours. After the 14-hour period, 500µL of Amp was added to six 2L flasks that contained 500mL of LB. An overnight starter culture was pre-grown and 25mL of the pre-grown starter culture was added to each 2L flask. These six 2L flasks were then incubated at 250rpm at 37°C (for about 2 hours) until the optical density of the medium reached 0.4-0.6. After the 6 flasks reached the 0.4-0.6 OD window they were then induced with 500mL of 1M Isopropyl β-D-1-thiogalactopyranoside (IPTG), a chemically analogous mimic of allolactose. Because IPTG is chemically similar to allolactose, IPTG triggers expression of the lac operon. *Lac operon* induction is of particular importance because our introduced G134E containing plasmid inserts its genetic information downstream and in close proximity of the *lac operon*. Therefore, IPTG induces the expression of G134E-

FGF1 in the BL21 (DE3) super competent *E. coli* cells. OD was then checked at an absorbance wavelength of 600nm using UV-vis spectroscopy. The contents of the six 2L flasks were then divided among centrifuge tubes and were centrifuged at 6,000rpm for 15 minutes. Immediately after centrifugation the supernatant was discarded to prevent pellet and supernatant re-mixing and the pellets were resuspended in 1xPBS pH 7.2 buffer. After the pellets were resuspended in PBS buffer, they were then aliquoted into 50mL flacon tubes and centrifuged one last time for 15 minutes at 6,000rpm. Immediately after centrifugation the supernatants were discarded and the pellets were stored in a -20°C freezer for future use.

#### *Purification of G134E-FGF1:*

To purify G134E-FGF1, an affinity chromatographic heparin-Sepharose column (~20ml bed capacity) was used. The bacterial pellets were removed from the -20°C freezer then thawed. The pellets containing the G134E-FGF1 protein were resuspended in 1xPBS pH 7.2 buffer containing 10mM NaCl and were then ultrasonicated while on ice for 3 sets of 20 cycles of alternating 10 second pulses/10 seconds resting periods with a power of 15 watts output. In addition, after each set of sonication 2 minutes of rest was given to the solution to allow it to cool. The sonicated solution containing the lysed bacterial cells were then centrifuged at 19,000rpm for 20 minutes. The clear supernatant containing G134E-FGF1 was immediately separated from from the pellet and loaded onto the pre-equilibrated heparin-Sepharose column with 10mM NaCl containing 10mM PB. After the supernatant was equilibrated on the heparin-Sepharose column (for 30 minutes) a salt gradient flow-through wash of 10mM PB and 100mM NaCl was passed

through the column. Next, washes of 500mM NaCl containing 10mM PB, 800mM NaCl containing 10mM PB, and 1500mM NaCl containing 10mM PB were used to wash proteins from the heparin-Sepharose column, with pure G134E-FGF1 eluting at 1500mM NaCl containing 10mM PB. The 1500mM NaCl solution passed through the column containing G134E was then dialyzed in dialysis tubing with a molecular weight cutoff of 3,500kDa. The last step was concentrating down pure G134E in a Millipore Ultra-15 centrifugal filter concentrator to achieve a final concentration of 1.5mg/mL then storing it in a -20°C freezer.

#### *Limited Proteolytic Trypsin Digestion:*

To compare the differences in resistance to proteolytic degradation of wild-type FGF1 (wtFGF1) to G134E-FGF1 limited proteolytic trypsin digestion was used as a probe with both proteins, wtFGF1 and G134E, in the presence and absence of heparin. The proteolytic degradation of wtFGF1 and G134E by trypsin was performed in a water bath at 37°C where trichloroacetic acid (TCA) was added to reaction mixtures to terminate the trypsin enzymatic activity. Two separate reaction mixtures for both wtFGF and G134E were prepared using 1mL of 0.005mg/mL solution of trypsin purified from a bovine pancreas along with 0.5mg/mL protein in 100mM NaCl containing 10mM PB. Two additional reaction mixtures were created (giving 4 total reaction mixtures) which mimicked the prior reaction mixtures but also contained 0.05mg/mL concentrated heparin. Immediately after all reaction mixtures were made, 100μL of each reaction mixture was taken from each sample and put on ice and 10μL of TCA was added to stop the reaction. This now 110μL solution represented the time zero sample with no (or

negligible) trypsin digestion. The reaction mixtures then incubated in the hot water bath for 5 minute intervals where at the end of each 5-minute period 100 $\mu$ L of the reaction mixture was removed, put on ice, and the reaction was halted with 10 $\mu$ L of TCA. The prior step was repeated for 45 minutes with 5 minute interval until all the reaction mixtures for each sample were depleted. All the samples, representing varying levels of trypsin digestion over time, were then processed using established TCA protein precipitation procedure. Each sample was analyzed for the magnitude of trypsin digestion using SDS-PAGE and densitometric scanning analysis.

#### *Thermal Denaturation of G134E-FGF1:*

To assess the changes in thermodynamic stability of the G134E-FGF1 compared wtFGF1 a thermal denaturation analysis was conducted on a Jasco 1500 spectropolarimeter using a 31.25 $\mu$ M protein concentration in 10mM NaCl containing 10mM PB. Data obtained was subjected to smoothing function available from the manufacturer. Appropriate blank subtractions were carried out. The absorbance wavelength of 228nm was measured from the spectra and the values were plotted against temperature.

#### *Far-UV Circular Dichroism (CD) Spectroscopy of G134E-FGF1:*

Using a Jasco 1500 spectropolarimeter data was collected on 0.5mg/mL samples of wtFGF1 and G134E FGF1 with and without heparin. 100 $\mu$ L of the solution samples were added to a 0.2mm path-length quartz cell. The Jasco 1500 spectropolarimeter was set to a wavelength range of 190-250nm at a 50nm/min scan speed and was loaded with

the quartz cell containing protein samples. The data was averaged across 3 scans, smoothed, and the buffer signal was subtracted out all using the JASCO spectra analysis software. The procedure was also completed for with-heparin samples, with the exception of the solution mixture that contained a 1:10 protein to heparin ratio. To account for the excess heparin signal for the solutions in the presence of heparin, the heparin signal was subtracted out of the data.

#### *8-Anilinonaphthalene-1-sulfonic acid (ANS) Binding Assay:*

To examine any changes in three-dimensional structure resulting from the exposure of interior hydrophobic residues that the G134E mutant may display, ANS binding assay was used to compare wtFGF1 and G134E-FGF1 in the presence and absence of heparin. The ANS assay was performed using a Hitachi F-2500 spectrofluorometer at an excitation wavelength of 380nm and an emission wavelength range of 450-600nm. A stock solution of ANS was made so that for every 1 $\mu$ L addition of the stock ANS the protein sample would increase the ANS concentration by 20 $\mu$ M. The protein samples were then diluted to a 15 $\mu$ M concentration. Next an initial recording of relative fluorescence intensity (RFI) was taken at the fluorescent wavelength of ANS, 500nm. ANS was then added to the protein sample in 1 $\mu$ L increments and the RFI was recorded for 20 measurements until a final concentration of 400 $\mu$ M ANS was achieved.

#### *Intrinsic Fluorescence Spectroscopy of G134E-FGF1:*

To investigate changes in the three-dimensional structure of the G134E mutant compared to wtFGF1 both in the presence and absence of heparin intrinsic fluoresce

spectroscopy was used. Samples of 0.1mg/mL of the respected protein in 100mM NaCl containing 10mM PB were prepared. The samples were then excited at a wavelength of 280nm and data was obtained from the range of 300-450nm to see if the intrinsic fluorescence of tyrosine (308nm) and tryptophan (350nm) were altered for the mutant compared to the wild type.

#### *Isothermal Titration Calorimetry (ITC) of G134E-FGF1:*

To measure changes in G134E's affinity to heparin compared to the wild type ITC was performed on a MicroCal ITC<sub>200</sub>. Solutions of 500μL containing 0.05mM G134E-FGF1 in PB buffer and 200μL of 0.5mM heparin in PB buffer were prepared. The data gathered was fitted with one set of sites binding model available on Origin software. In addition, excess heparin was subtracted and the binding affinity was determined by the dissociation constant ( $K_d$ ).

#### *Bioactivity Assay:*

To understand the effects of the G134E mutation on FGF1's ability to carry out cellular proliferation, two 50μL samples of 1mg/mL, one for both wtFGF1 and G134E, were prepared in 100mM NaCl containing PB. The bioactivity assay used NIH/3T3 cells with increasing concentrations of protein up to 3nM to measure cellular proliferation for the wild type and G134E-FGF1.

## **Results and Discussion:**

### *Isolation of G134E-FGF1 on heparin-Sepharose affinity chromatography:*

To perform the biophysical characterization of the G134E mutant, G134E-FGF1 BL21-DE3 cells were transformed with pET20b-G134E-FGF1 mutant plasmid. After transformation, the bacterial cells housing the G134E mutated protein were then overexpressed in a nutrient rich LB medium. Successful expression of the G134E mutant was then verified using 15% SDS-PAGE analysis.

After generating the G134E mutant, pure G134E-FGF1 was isolated on the heparin-Sepharose column using a phosphate buffer/NaCl salt gradient of increasing NaCl concentrations from: 500mM NaCl, 800mM NaCl, and 1500mM NaCl with purified FGF-1 eluting at 1500mM NaCl. Because of FGF1's characteristic affinity to heparin, other cellular proteins and cell contents were washed from the heparin-Sepharose column at the low salt concentrations. Wild type FGF1 is known to elute off of heparin-Sepharose at 1500mM NaCl because at this strength of NaCl, FGF1's electrostatic interactions with heparin-Sepharose would completely break apart to release the bound FGF1 from the resin. SDS-PAGE analysis shows that like the wild type FGF1, G134E also eluted from the column at 1500mM NaCl concentration. Collecting the G134E at a 1500mM NaCl salt concentration, serves as conformation that the G134E mutation does not significantly alter FGF1 surface residues and its inherent affinity to heparin. Overexpression and purification of G134E-FGF1 was analyzed by SDS-PAGE analysis.

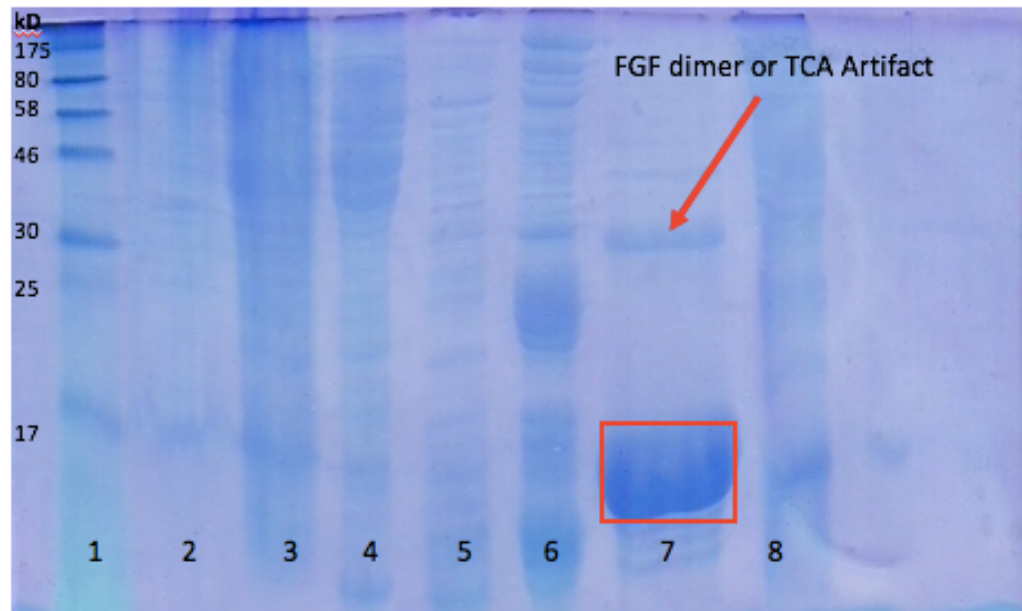


Figure 9: SDS-PAGE analysis of G134E-FGF1 purification fractions collected from heparin-Sepharose column: Lane-1: Protein marker, Lane-2: insoluble cell debris, Lane-3: clear cell lysate, Lane-4: flow-through of 100mM NaCl 10mM phosphate buffer, Lane-5: 500mM NaCl 10mM phosphate buffer, Lane-6: 800mM NaCl 10mM phosphate buffer, Lane-7: 1500mM NaCl 10mM phosphate buffer, Lane-8: 8M Urea wash to clean the column. The bottom band, boxed in red in lane-7 corresponding to 16kD position, confirms the presence of G134E FGF-1. The band in lane-7 at the 30kD position is the result of two possibilities: a G134E dimer or a TCA artifact.

#### *Determination of heparin binding affinity G134E-FGF1 mutant using isothermal titration calorimetry:*

ITC was used to measure the dissociation constant ( $K_d$ ) of heparin to the wild type FGF-1 and to the G134E mutant. Both the G134E-FGF1 mutant and the wild-type dissociation constants were calculated. A higher  $K_d$  is indicative of a weaker interaction and less affinity between two molecules, in our case heparin and FGF1, where a lower  $K_d$  signifies a tighter binding interaction between two molecules. Compared to the wild type FGF1, G134E-FGF1 had a higher  $K_d$  (wild type FGF1's  $K_d = 0.316 \mu\text{M}$ ; G134E-FGF1's  $K_d = 6.67 \mu\text{M}$ ) which corresponds to G134E having a moderately decreased affinity to

heparin which is in agreement with the hypothesis of introduction of negative charge in the heparin interaction region results in decreased binding affinity.

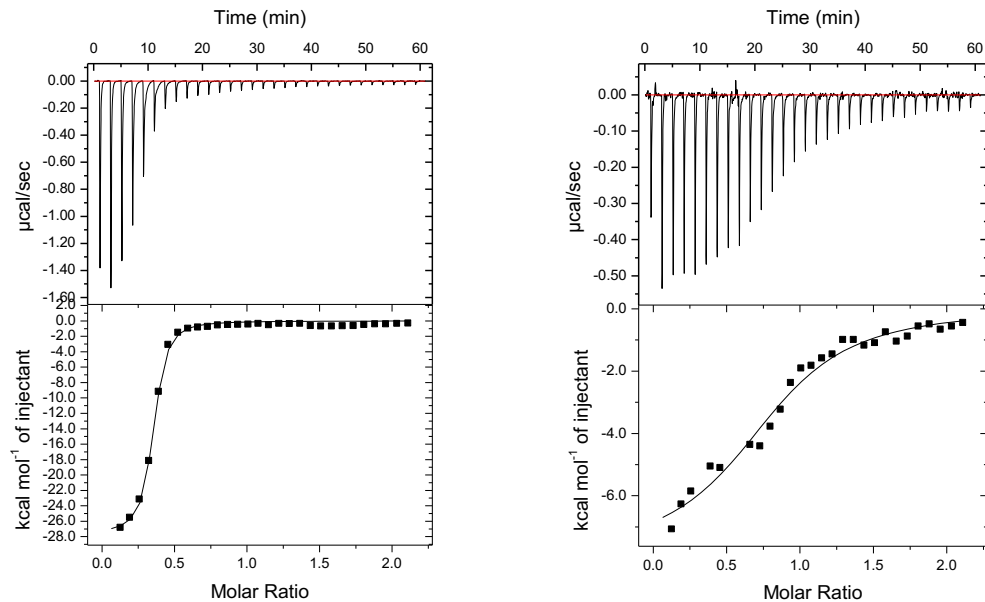


Figure 10: Isothermograms of wild type FGF1 (left) and G134E mutant (right) showing their respective interaction to heparin. The bottom panel represents the best-fit of the raw data using one-set of sites binding model available from Origin software. Dissociation constants ( $K_d$ ) of 6.67  $\mu$ M (G134E) and 0.316  $\mu$ M (wtFGF1) were calculated.

#### *Determination of stability of G134E-FGF1 by limited proteolytic digestion assay:*

To assess G134E-FGF1's resistance to proteolytic cleavage, G134E was subjected to limited trypsin digestion followed by SDS-PAGE analysis and densitometric scanning. By comparing the assays of G134E-FGF1 to that of wtFGF1, we may assess the affects that this point mutation has on the resistance to proteolytic degradation of FGF1. Trypsin was our protease of choice because it is not a specialized protease limited by consensus sequences. Rather, trypsin cleaves with general selectivity at the carboxyl end of lysine and arginine residues. This generalized cleavage of trypsin allows us to achieve a more

uniform proteolytic activity over time which is useful to characterize the intrinsic structural stability and resistance to proteolytic degradation of the G134E mutant.

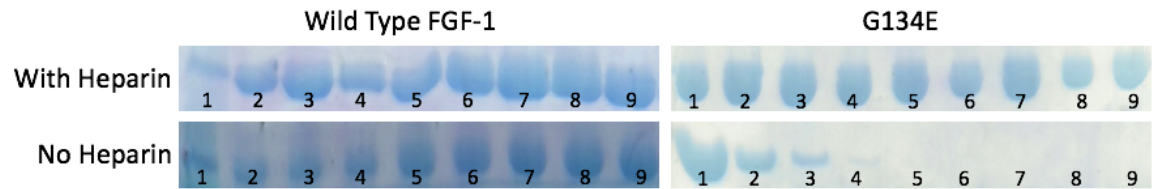


Figure 11: SDS PAGE analyses of the limited trypsin digestion products of the G134E mutant compared to that of wild type FGF1 in the presence and absence of heparin. Lane-1 corresponds to the time zero sample before trypsin addition. Each subsequent lane (lanes 2-10) represent a 100  $\mu$ L extraction from the trypsin reaction collected and then halted via addition of trichloroacetic acid (TCA) at 5 minute intervals, with lane 10 concluding the assay at 45 minutes. Lane-1: time-zero sample, Lane-2: 5 minutes, Lane-3: 10 minutes, Lane-4: 15 minutes, Lane-5: 20 minutes, Lane-6: 25 minutes, Lane-7: 30 minutes, Lane-8: 35 minutes, Lane-9: 40 minutes.

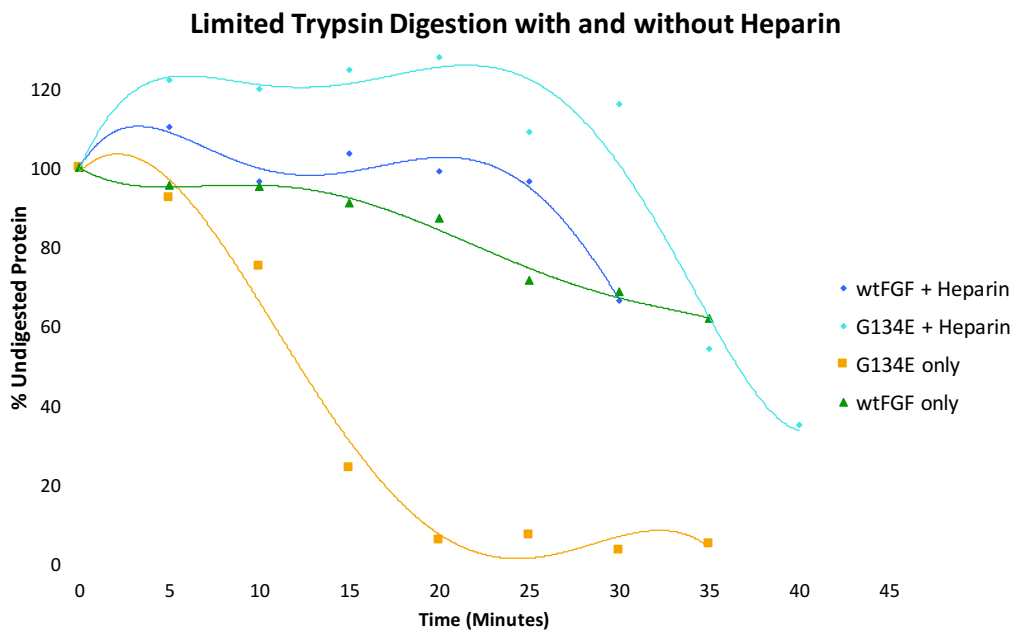


Figure 12: Densitometric scan of the undigested wtFGF1 and G134E over time in the presence and absence of heparin after treatment of trypsin. Lines of best fit are plotted through each data set. The data sets are represented as follows: wtFGF1 (dark blue) in the presence of heparin; G134E-FGF1 (light blue) in the presence of heparin; G134E-FGF1 only (orange), without heparin; and wtFGF1 only (green) in the absence of heparin.

Both the wild type FGF1 and G134E's with-heparin plots are relatively stabilized by the presence of heparin. This lends support to heparin acting as a shield to proteolytic

degradation. Though wtFGF1 in the absence of heparin shows increased proteolytic degradation compared to when it is bound to heparin, the G134E mutant is characterized with a substantial rate of digestion—over 90% of the G134E only mutant was degraded after 20 minutes of trypsin exposure in the absence of heparin whereas approximately 20% of the wild type FGF-1 protein was cleaved. This data shows that G134E-FGF1 without heparin is highly susceptible to trypsin degradation compared to that of the wild type. The increased susceptibility of the G134E mutation could be the result of the introduction of a negatively charged amino acid causing the protein core to be less tightly packed. An unpacked core would allow for trypsin to act upon more of its lysine and arginine targets consequently causing a greater digestion rate.

*Ascertaining surface hydrophobicity of G134E-FGF1 mutant:*

ANS titration was performed to assess the surface hydrophobic differences between the G134E mutant and wild type FGF1. By measuring the fluorescence of bound ANS to the exposed hydrophobic pockets the folding and flexibility of G134E can be compared to wtFGF1. ANS is a fluorescent, hydrophobic dye that gives a signal when it binds to exposed hydrophobic pockets on the surface of protein (Zorrilla et al. 2010). Specifically, ANS lends information of a mutant proteins' susceptibility to aggregation by a greater fluorescence emission denoting a movement of hydrophobic residues from the hydrophobic interior of the protein to the hydrophilic exterior.

Comparisons between the global structure of G134E and wtFGF1 can be assessed by comparing the ANS data. The data reveals that when increasing the ANS concentrations both wtFGF1 and G134E mutant follows the same trend both with and

without heparin. This indicates that the G134E mutant core is packed in a similar fashion to the wild type. Therefore, a similar amount of hydrophobic residues are exposed between G134E and wtFGF1. This data also indicates that the folding and flexibility of G134E-FGF1 is similar to that of the wild type. Additionally, this data suggests that the G134E appears to render the protein more prone to aggregation.

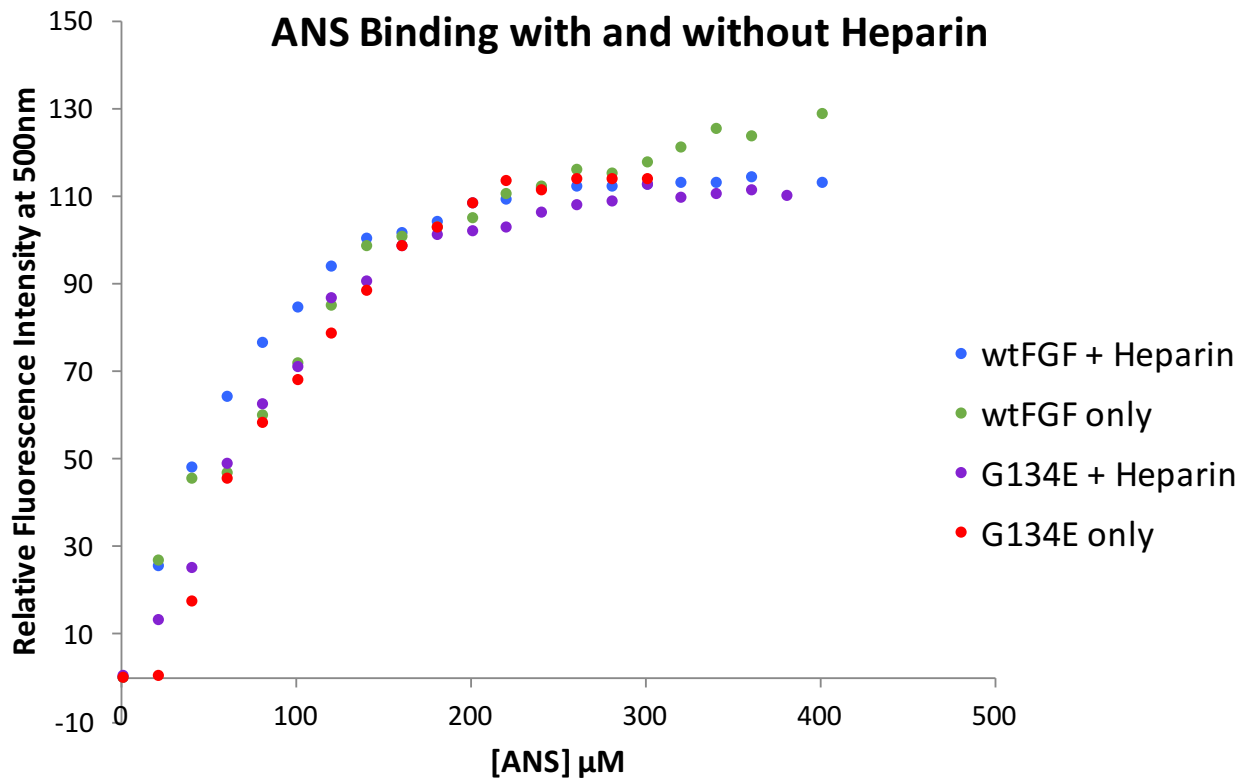
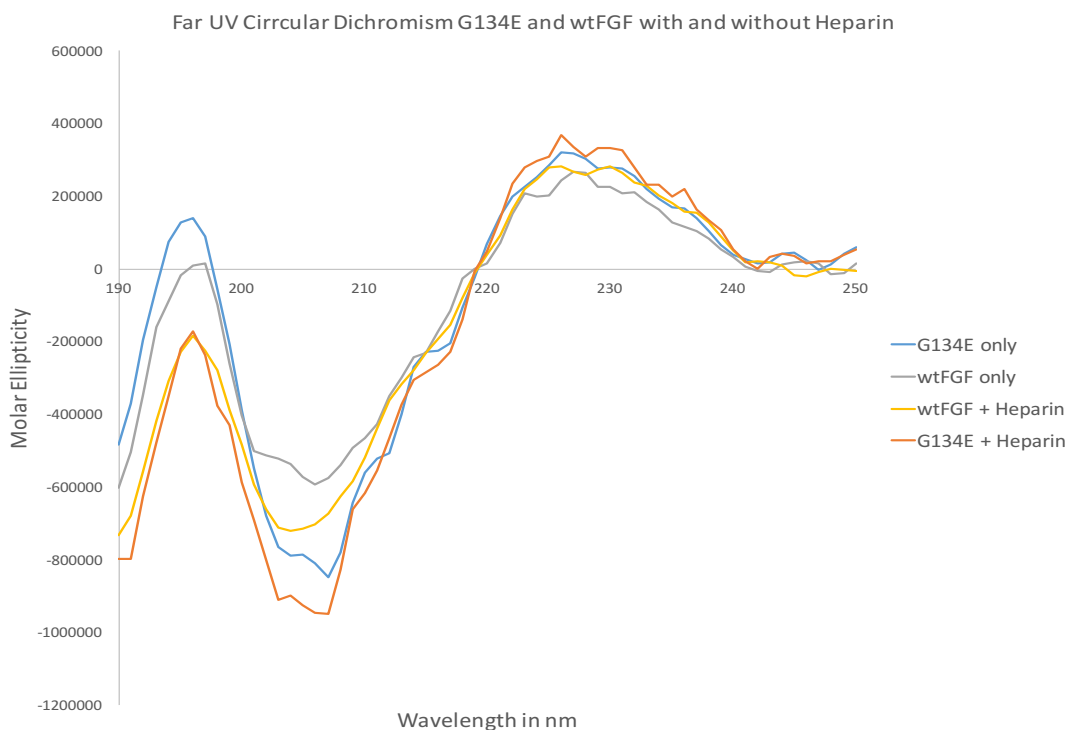


Figure 13: ANS titration of wild type FGF1 and G134E mutant in the presence and absence of heparin.

*Determining the changes in the secondary structure of G134E-FGF1 mutant:*

The hallmark CD spectra of a  $\beta$ -sheet is known to be positive from 190nm to approximately 210 nm then negative for the remainder of the plot. However, because FGF1 is arranged in a  $\beta$ -barrel (more precisely, a  $\beta$ -trefoil fold), the dipoles of the  $\beta$ -sheets are cancelled. Consequently, the CD spectra of a  $\beta$ -barrel is different from that of a  $\beta$ -sheet where the far UV CD plot for a  $\beta$ -barrel has a strong positive peak at 228nm—indicative of a  $\beta$ -barrel. Using polarized light, the secondary structure of G134E is analyzed compared to wtFGF1 with and without the presence of heparin. Because the peak of 228 is clearly visible for the mutant plots, both with and without heparin, the CD assay reveals that the G134E mutation does not perturb secondary structure and the  $\beta$ -barrel of FGF1 is left intact; therefore, secondary structure for G134E is upheld and similar to that of the wild type.



*Figure 14: Overlay of far UV CD spectra of the wild type and G134E mutant of FGF1, in the presence and absence of heparin.*

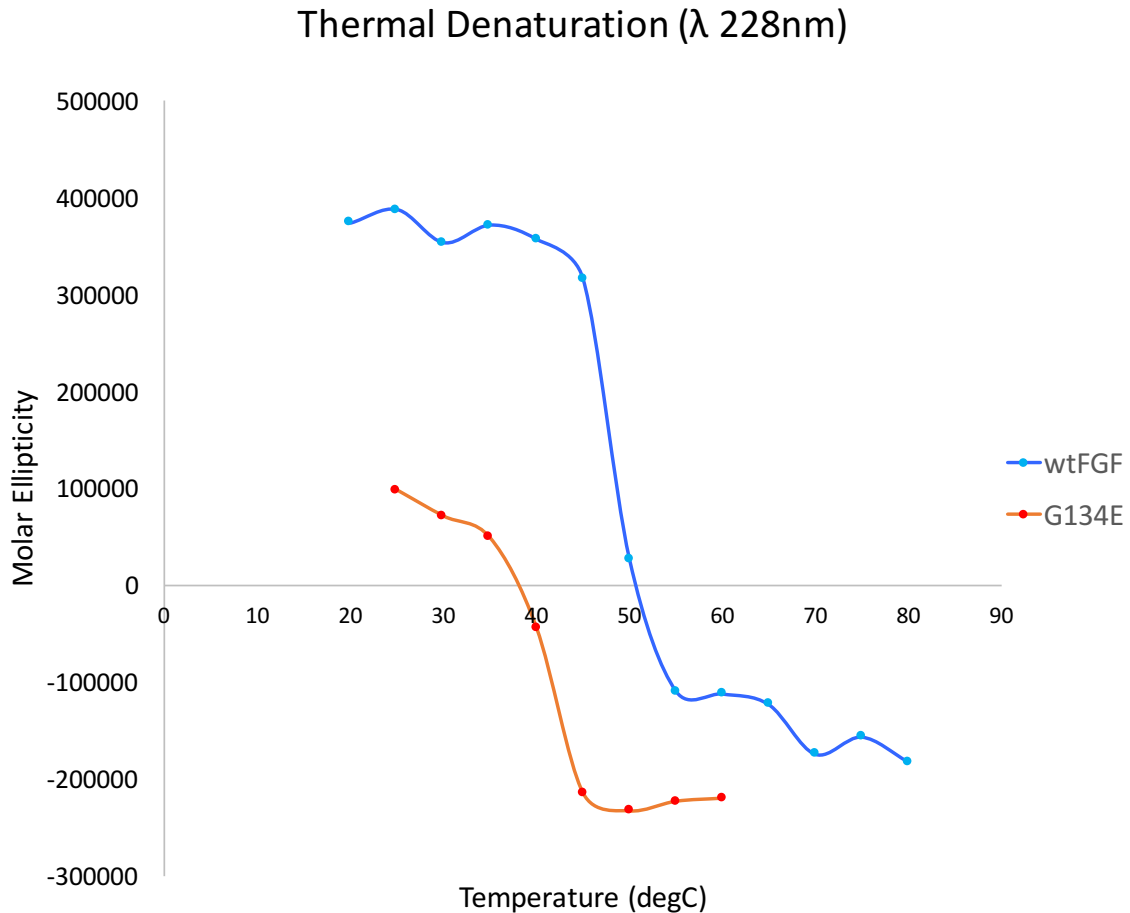


Figure 15: Thermal denaturation profile of the wild type and G134E mutant of FGF1 in the presence and absence of heparin monitored by 228 nm molar ellipticity.

Because the  $\beta$ -barrel is observable at 228nm, thermal denaturation was observed at a wavelength of 228nm to assess the thermal stability of the  $\beta$ -barrel consequently yielding the thermal stability of the entire protein. Both thermal denaturation temperatures of wild type FGF1 and G134E were measured by finding the average value of molar ellipticity values then corresponding the average molar ellipticity to a temperature. The denaturation temperature of wild type FGF1 was measured to be 49 °C where G134E's thermal denaturation temperature was 41 °C. The lower melting temperature ( $T_m$ ) of the G134E mutant is describing that G134E loses local integrity of secondary structure at a lower temperature. Although the G134E does not perturb the global secondary structure

and the  $\beta$ -barrel is upheld, the G134E mutant is thermodynamically less stable than the wild type and G134E denatures at a lower temperature which is consistent with the trypsin digestion data.

*Determination of changes in tertiary structure of G134E-FGF1 using intrinsic fluorescence as a probe:*

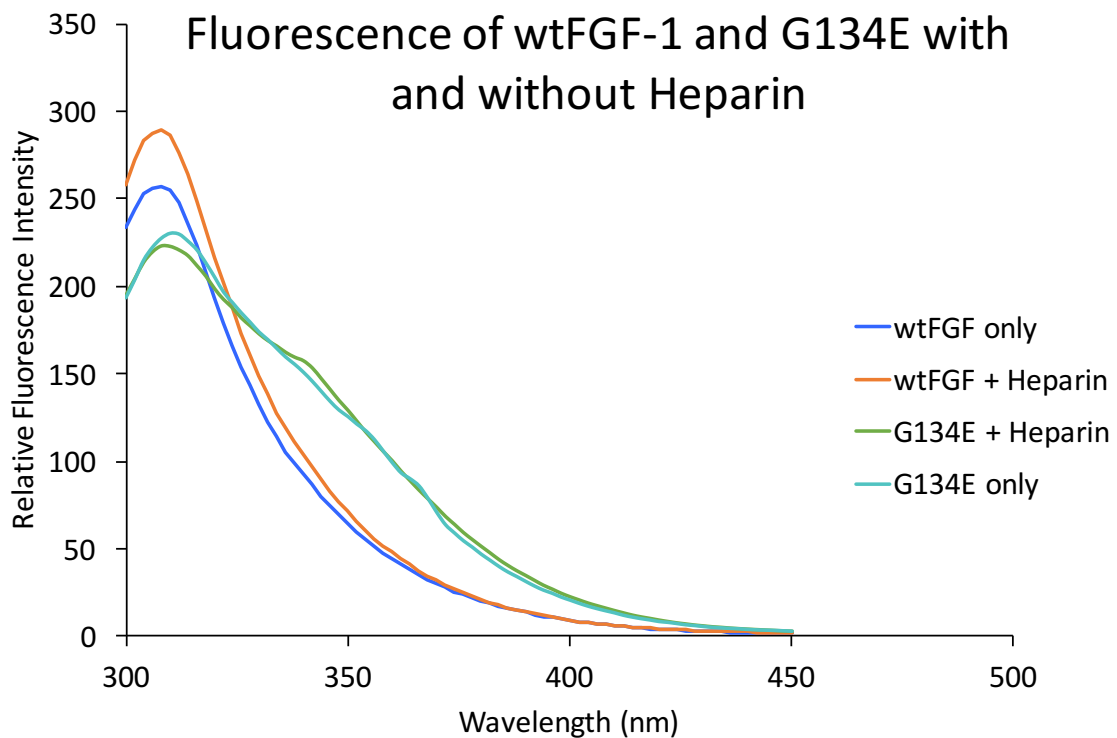


Figure 16: Overlay of intrinsic fluorescence spectra of wild type FGF1 and G134E mutant in the presence and absence of heparin.

To understand changes in the tertiary structure of the G134E mutant compared to that of the wild type in the presence and absence of heparin intrinsic fluorescence measurement was performed. The peak for all data sets at approximately 308nm is indicative of tyrosine fluorescence, a signature pattern for FGF1. The intrinsic fluorescence spectra

reveals that the G134E mutant induces a solvent exposed tryptophan in both the presence and absence of heparin due to the shouldering of the G134E plots at 350nm.

*Effect of G134E mutation on the biological activity of FGF1:*

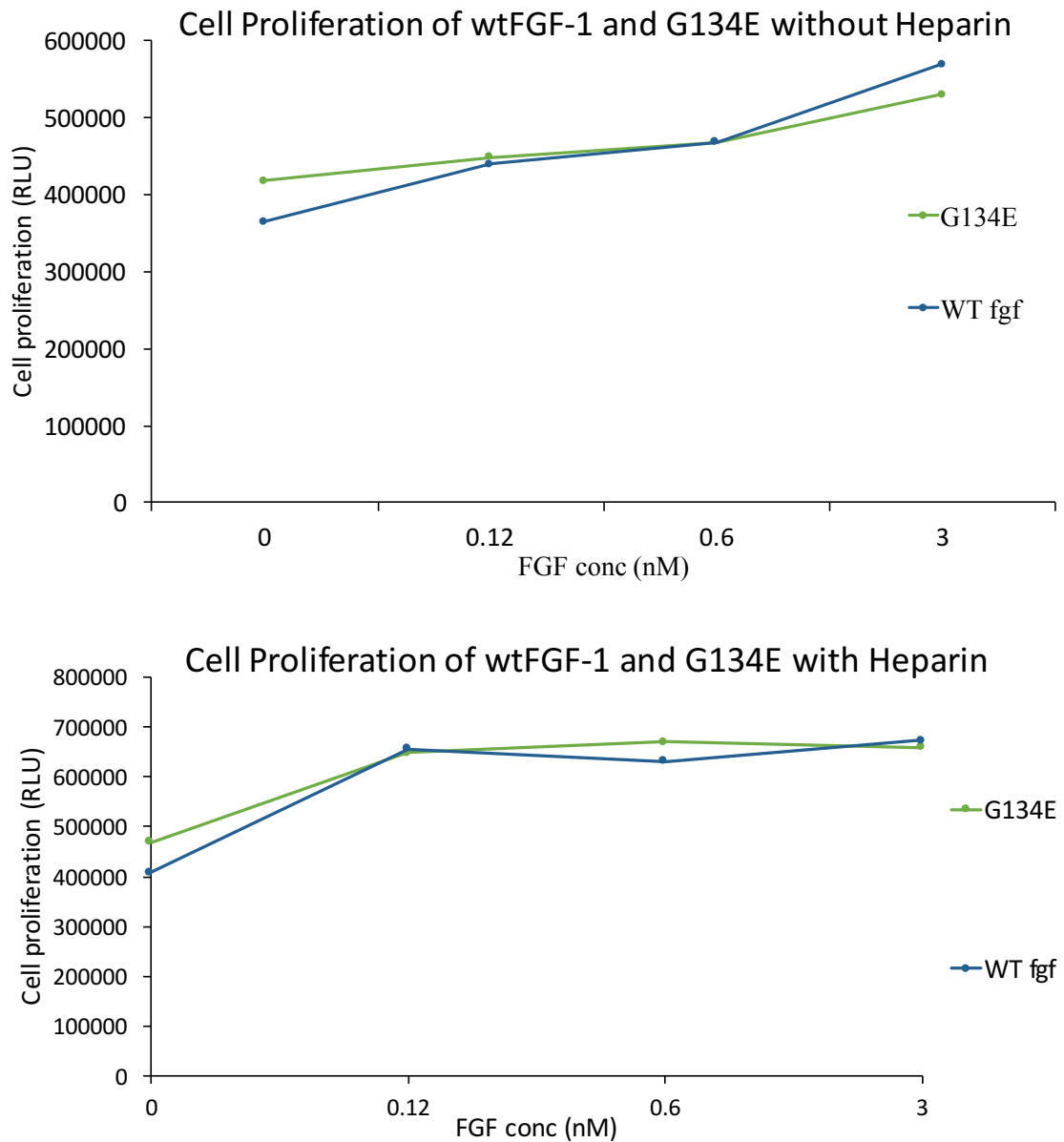


Figure 17: Cell proliferation activity of the wild type FGF1 and G134E mutation with heparin (bottom graph) and without heparin (top graph).

In both the presence and absence of heparin the G134E mutation has similar cell proliferation rates as the wild type. In the presence of heparin, G134E causes just slightly more or equal cell proliferation at almost all concentrations. In the absence of heparin, G134E causes slightly more cell proliferation than the wild type up until 0.6nM concentration.

### **Conclusions and Future Work:**

The biophysical characterization showed that the G134E-FGF1 mutant induces minor yet noteworthy changes to the human acidic FGF1. ITC results showed that G134E mutation resulted in decreased binding affinity to heparin with a higher dissociation constant ( $K_d$ ) compared to the wild type. Although G134E's innate propensity to heparin was moderately reduced, G134E-FGF1 still strongly associates with heparin. This was proven by successful purifications of G134E where 1500mM NaCl was required to disrupt the interaction between G134E-FGF1 and heparin. Limited trypsin digestion of G134E suggests that introduction of a negative charge in the heparin binding pocket renders heparin to not efficiently confer additional resistance against trypsin digestion.

Although CD data supports that secondary structure of G134E is not compromised and the  $\beta$ -barrel is still intact, the introduction of negatively charged glutamic acid appears to increase the conformational flexibility as compared to the wild type FGF1. This inference is supported by intrinsic fluorescence and thermal denaturation wherein the  $\beta$ -barrel structure of the G134E mutant is observed to be disrupted at a lower temperature than the wild type FGF1. In addition, results of the ANS binding

experiments suggest that the G135E mutation appears to render the protein more prone to aggregation than the wild type FGF1. The evidence supports that the structural integrity of G134E is well preserved but minor structural perturbation induced by the introduction of the negatively charged glutamic acid causes the protein to be marginally less thermodynamically stable.

Interestingly, the induced structural changes, caused due to the mutation, do not appear to alter the mitogenic activity of the growth factor.

In regard to future work, the Kumar lab will carry out multidimensional NMR spectroscopy experiments which are likely to provide atomic level information of the subtle conformational changes caused by the G135E mutation. Further studies to determine G134E's affinity towards varying FGFRs isoforms using ITC experimentation will provide information on the binding affinity between the mutant protein and the receptor.

The results of this study are likely to aid future studies, utilizing point mutations of FGF1, to understand and characterize the residues that impact the stability of FGF1. In particular, the results of this study provide interesting leads in understanding the structural forces responsible for the FGF-heparin interaction

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