CROSS TALK BETWEEN FANCONI ANEMIA AND UNC5A SIGNALING PATHWAY

Thèse

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RÉSUMÉ

L'anémie de Fanconi (AF) est une maladie infantile multigénique et complexe. Les enfants atteints d'AF souffrent d'une insuffisance médullaire progressive potentiellement mortelle. En plus du phénotype hématologique, les enfants souffrant d'AF présentent de nombreuses malformations congénitales incluant le système nerveux central et une prédisposition accrue aux cancers particulièrement de type leucémique. Plusieurs gènes associés à la maladie ont été identifiés mais leur fonction dans l'étiologie de la maladie demeure inconnue. La présence d'une mutation dans l'un des gènes Fanconi entraine une perte progressive des cellules souches hématopoïétiques (CSH) menant à un épuisement médullaire et favorisant l'apparition de leucémies. Les protéines Fanconi forment trois complexes protéiques distincts qui participent de manière séquentielle dans une voie de signalisation en réponse aux dommages à l'ADN. La protéine *Fanconi Anemia* de groupe C, ou FANCC, est une composante du complexe majeur de cette voie Fanconi. Outre son rôle dans la voie Fanconi et dans les mécanismes de signalisation en réponse aux dommages à l'ADN, FANCC est connue pour son implication dans la mort cellulaire programmée, la détoxification des radicaux oxygénés et la réponse aux cytokines. Afin d'identifier la fonction de la protéine FANCC dans les mécanismes de développement, nous avons procédé à un criblage d'une banque d'ADNc et identifié certains partenaires biochimiques de FANCC tel le récepteur de la Netrine-1, uncoordinated-5A (UNC5A). Puisque le récepteur UNC5A a une fonction de signal de survie cellulaire et est impliqué dans les mécanismes de croissance neuronale, nous avons étudié le rôle de l'interaction

FANCC-UNC5A dans les mécanismes de différenciation neuronale. Nos résultats indiquent que FANCC régule la fonction pro-apoptotique de UNC5A. Lorsque FANCC est surexprimée, les cellules retardent leur entrée en apoptose tandis qu'en absence de FANCC, UNC5A favorise l'entrée en apoptose. De plus, nos résultats indiquent que FANCC conjointement à UNC5A promeut la neurogénèse; FANCC et UNC5A colocalisent dans les neurites cellulaires. Globalement, nos résultats suggèrent que FANCC par le biais de UNC5A joue un rôle important dans la mort cellulaire et la croissance axonale. Ainsi, une dérégulation de l'interaction FANCC-UNC5A chez les patients souffrent de FA pourrait expliquer certains aspects cliniques notamment les anomalies de développement.

ABSTRACT

Fanconi anemia (FA) is a recessive syndrome characterized by diverse clinical symptoms including progressive bone marrow failure, various congenital abnormalities, chromosomal instability and predisposition to malignancies. Studies of the canonical FA pathway have focused on the mechanism of repair of DNA cross-linking damage. However, some data suggest that FA proteins may have other functions besides DNA damage signaling events, and these functions may explain some of the disease phenotypes such as defects in hematopoiesis and congenital malformations. For instance, FANCC, which is predominantly located in the cytoplasm, has multifunctional roles and is an anti-apoptotic regulator. In addition to its function as a repulsive mediator in neural development, UNC5A, the receptor for the axon guidance molecule Netrin-1, has also been proposed to be a “dependence receptor” that triggers apoptosis in the absence of its ligand. Here, we identified a novel interaction of UNC5A with FANCC and showed that FANCC positively regulates UNC5A-mediated apoptosis. Under conditions of FANCC overexpression, apoptosis is decreased, whereas the absence of a functional FANCC protein increases UNC5A-mediated apoptosis. Furthermore, FANCC and UNC5A function as a complex in neurogenesis; they co-localize at synapses formed by neurites, and FANCC is required for the promotion of neuronal outgrowth by UNC5A. Based on these findings, we propose that FANCC plays a key role in tissue morphogenesis by either delaying UNC5A-mediated apoptosis or positively impacting the expression of UNC5A. Under FANCC-deficient conditions, dysregulation of the UNC5A signal pathway can lead to developmental defects such as those seen in FA patients.

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ABBREVIATIONS

Units of measurement

M	molar
mM	millimolar
μM	micromolar
mg	milligram
μg	microgram
mL	milliliter
μL	microliter
kDa	kilodalton
MDa	megadalton

Abbreviations

α	alpha
a.a.	amino acid
AA	aplastic anaemia
AIY	interneurons integrating the receptors of the amphid sensilla
ALL	Acute Lymphoid Leukemia
AML	Acute Myeloid Leukemia
APS	Ammonium persulfate
ATM	Ataxia Telangiectasia Mutated protein kinase
ATR	Ataxia Telangiectasia and RAD3 related protein
β	beta
BACE	Beta site APP cleaving Enzyme
BACH1	BRCA1 Associated C-terminal helicase 1
BRCA	Breast Cancer
BRIP1	BRCA1 Interacting Protein 1
BM	Bone Marrow
BMF	Bone Marrow Failure
BMT	Bone Marrow Transplant
BSA	Bovine serum albumin
C.elegans	*Caenorhabditis elegans*
CD34	Cluster of Differentiation 34
Cdc2	Cyclin-dependent kinase 1
CNS	Central Nervous System
CO_2	Carbon dioxide
C-terminal	Carboxy terminal
DA9	Ventral cord motor neurons, innervate dorsal muscles
DAPK	Death-Associated Protein Kinase
DCC	Deleted in Colorectal Cancer
DD	Death domain

DEB	Diepoxybutane
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ECL	Enhanced Chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal Growth Factor
FA	Fanconi Anemia
FANC	Fanconi Anemia Protein (from FANCA to FANCP)
FACS	Fluorescence-activated cell sorting
FAZF	Fanconi Anemia Zinc Finger protein
FBS	Fetal Bovine Serum
γ	gamma
G1/G2	growth phase 1/growth phase 2
GM-CSF	Granulocyte/Macrophage Colony Stimulating Factors
GRP94	Glucose-Related Protein 94
GSTP1	Glutathione S-Transférase P1
HA	Human influenza hemagglutinin
HEK 293T	Human Embryonic Kidney 293T cell
HeLa	Human epithelial carcinoma cell line
HES	Hairy and Enhancer of Split
HR	Recombinaison Homologue
HSC	Hematopoietic Stem Cell
Hsp	Heat shock protein
H.sapiens	*Homo sapiens*
ICLs	DNA interstrand crosslinking agents
IFN	Interferon
IgG	Immunoglobulin
IL	interleukin
IFAR	International FA Registry
IP	Immunoprecipitation
JAK	Janus Kinase
JNK	c-Jun-N-terminal kinase
M	Mitosis phase
MAC	mitochondrial apoptosis-induced channel
MDS	Myelodysplasic Syndrome
MMC	Mitomycine C
mRNA	messenger RNA
NAD(P)(H)	Nicotinamide Adenine Dinucleotide Phosphate reduced form
NBS1	Nijmegen Breakage Sydrome 1 protein
NER	Nucleotide Excision Repair
NES	Nuclear Export Localization Signal
NHEJ	non-homologuous end-joining
NLS	Nuclear Localization Signal

N-proximal	amino proximal
NRAGE	Neurotrophin receptor-interacting melanoma antigen homolog
PALB2	Partner And Localizer of BRCA2
PBS	Phosphate Buffered Saline
PCD	programmed cell death
PCR	Polymerase Chain Reaction
PEST	Proline/Glutamic acid/Serine/Threonine-rich
PHD	Plant Homeodomain
PHF9	PHD Finger protein 9
PKR	double stranded RNA dependent protein kinase
PMFS	Methyl phenyl sulfoxide
pRb	Retinoblastoma protein
PVDF	polyvinylidene difluoride
RAD50/51	RecA homologue
RMN	RAD50/MRE11/NBS1
RNA	Ribonucleic acid
RNA pol II	RNA polymerase II
ROS	Reactive Oxygen Species
RPA1	Replication Protein A1
RPM	rotation per minute
RT	Room Temperature
S.cerevisiae	*Saccharomyces cerevisiae*
SCC	Squamous Cell Carcinoma
SD	Synthetic Dropout
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	sodium dodecylsulfate-polyacrylamide gel electrophoresis
siRNA	Short Interference RNA
SMACs	small mitochondria derived activator of caspases
STAT-1	Signal Transduction and Activator of Transcription protein 1
TBS(T)	Tris Buffered saline (tween)
TEMED	N,N,N',N'-Tetramethylethylenediamine
TGF	Transforming Growth Factor
TGIF	TGFB (transforming growth factor beta) induced factor
TLE	Transducin-Like Enhancer of split
TLS	Translesion Synthesis
TNF	Tumor Necrosis Factor
TRAIL	TNF-related apoptosis-inducing ligand
Ub	Ubiquitin
Unc5	Uncoordinated-5
USP1	ubiquitin Specific Peptidase 1
WCE	Whole cell extract
WT	wildtype
XIAP	X-chromosome-linked inhibitor of apoptosis protein
X.laevis	*Xenopus laevis*

X.tropicalis	*Xenopus tropicalis*
XRCC9	X-ray Repair, Complementing defective in Chinese hamster, 9
YPAD	Yeast extract-peptone-adenine-dextrose medium
ZU-5	Zona Occludens-1 homology domain

ACKNOWLEDGEMENT

Foremost, I would like to express my sincere gratitude to my advisor, Dr. Madeleine Carreau, for her continuous support of my Ph.D. study and research. After I completed my graduation study in China, further education in the West was one of my dreams. Dr. Madeleine provided me with this opportunity and helped me realize my dream. She opened the door for me to enter the research field of molecular and cellular biology. Her patience, motivation, enthusiasm, and immense knowledge guided and helped me throughout my research and the writing of this thesis. Especially during my two pregnancies, she was so patient and continually encouraged me to achieve my Ph.D. I could not have imagined having a better advisor and mentor for my Ph.D. study.

I would also like to thank Dr. Georges Lévesque, one of the best geneticists I have known. He was always there and generously provided his help. His solid scientific knowledge always helped me with difficulties I encountered in my project.

I would like to thank my colleagues in the groups of Dr. Madeleine Carreau and Dr. Georges Lévesque: Marie-Chantal Delisle, Tagrid Kaddar, Caroline Huard, Audrey Magron, Chantal Godin, Carolina Koutras, Manel Ben Aïssa and Kevin Goggin. I express my sincere thanks for their suggestions and help during my research.

Furthermore, I would like to express special thanks to my husband, Peidong, for his love and support throughout my Ph.D. study. Also, I give thanks to and for my two babies, who are the energy for my work, especially at the end of my thesis writing.

Finally, I would like to thank my families in China for their understanding and support from so far away. I am grateful to my mom for staying healthy and coming to visit me in Canada and to my brother and sister and their families for caring about my parents and for supporting me. This thesis is also a special commemoration of my father.

FOREWORD

This thesis is based on Ph.D. research on the functional connection between Fanconi anemia, especially FANCC protein, and the UNC5A signaling pathway. In 2006, Caroline Huard identified UNC5A as a potential partner of FANCC protein in yeast 2-hybrid screening experiments. This finding led us to further investigate the possible function of UNC5A in the FA pathway and the role of FANCC in the UNC5A signaling pathway. With the help of my supervisor, Dr. Madeleine Carreau, and my colleagues, I continued and finished most parts of this project.

The thesis consists of 5 chapters: introduction, hypothesis and objectives, results, discussion and conclusion. Two articles describing a major part of my results have been published and are included as a major part of my work in the results chapters. Manel Ben Aissa, Audrey Magron, Caroline C. Huard, Chantal Godin and Georges Lévesque all contributed to my first paper. I would like to thank François Marcouiller from the Neuroscience Department for his work that forms part of my second paper.

This thesis is the memory to my Ph.D. research study, to my thousands of days of working in the lab, battling shoulder to shoulder with my colleagues and friends, the joy in the synthesis, the hope for good results and the sadness and tiredness that came with each failed attempt.

Finally, I wish you a good reading. Thank you.

CHAPTER 1: INTRODUCTION

1.1 Fanconi anemia

1.1.1 Definition

Fanconi anemia (FA) is a rare genetic disorder characterized by multiple developmental anomalies, hematological abnormalities and predisposition to a variety of cancers. It is named for the Swiss pediatrician Guido Fanconi (1892-1979), who described a family in which three sons suffered from various physical abnormalities such as short stature, microcephaly, intensive brown skin pigmentation, very lively tendon reflexes, and a condition that resembled pernicious anemia[1] . FA is primarily an autosomal recessive genetic disorder. This means that two mutated alleles (one from each parent) are required to cause the disease. There is a 25% risk that each subsequent child will have FA. Approximately 2% of FA cases are X-linked recessive, which means that if the mother carries one mutated FA allele there is a 50% chance that male offspring will present with FA. FA occurs equally in males and females, and it is found in all ethnic groups [2, 3]. The prevalence of FA is 1 to 5 cases per 1 million persons, and the heterozygous carrier frequency is approximately 1 per 300 persons. The median age at diagnosis is 6.5 years for boys and 8.0 years for girls, but the overall age at diagnosis ranges from 0 to 48 years. The current median lifespan for a patient with FA is 29 years, although there are now patients living into their 30s, 40s and 50s [4].

1.1.2 Clinical features

1.1.2.1 Developmental abnormalities

Individuals with FA display diverse developmental abnormalities ranging from physically normal (approximately 25 to 40%) to abnormalities severe enough that the pregnancy results in spontaneous abortion or prenatal lethality (Table 1.1) [5-7].

Table 1.1: Congenital malformation in patients in the International FA Registry (IFAR), adapted from Kutler, D.I., et al. Blood, 2003.

Congenital malformations	Frequency(%)	Clinical manifestations
Skin	55	Café-au-lait spots, hyper- and hypopigmentation
Growth	51	Intrauterine growth retardation, short stature, endocrine abnormalities
Eyes	23	Microophthalmia, short or almond shaped palpebral fissures, ptosis, epicanthal folds, hyper- and hypotelorism, strabismus, cataracts
Thumb and radius	50	Thenar hypoplasia, absence or hypoplasia of radius and/or thumb, floating thumb, bifid thumb, digitalized thumb/abnormal thumb placement
Other skeletal	21	Dysplastic or absent ulna, micrognathia, frontal bossing, spina bifida, Klippel-Feil, vertebral anomalies, absent clavicles, Sprengel's deformity, Perthes disease, congenital hip dysplasia/dislocation, scoliosis, rib abnormalities, clubfoot, sacral agenesis (hypoplasia), leg length discrepancy, kyphosis, brachydactyly, arachnodactyly, humeral abnormality, craniosynostosis
Kidney and urinary tract	21	Ectopic, horseshoe, rotated, hypoplastic or absent, dysplastic, hydronephrosis, hydroureter, urethral stenosis, reflux
Ears	22	Deafness (usually conductive), abnormal or absent pinna, prominent ears, abnormally positioned ears (low set or posteriorly rotated), small or absent ear canals, absent tympanic membrane, microtia, fused ossicles
Genital	20	Males: micropenis, penile/scrotal fusion, undescended or atrophic or absent testes, hypospadius, chordee, phimosis, azospermia Females: bicornate uterus, aplasia or hypoplasia of vagina and uterus, atresia of vagina, hypoplasic uterus, hypoplastic/absent ovary, hypoplastic/fused labia
Cardio-pulmonary	13	Patent ductus arteriosis, ventricular septal defect, pulmonic or aortic stenosis, coarcation of the aorta, double aortic arch, cardiomyopathy, tetralogy of Fallot, pulmonary atresia
Gastrointestinal	14	Esophageal atresia, duodenal atresia, anal atresia, tracheoesophageal fistula, annular pancreas, intestinal malrotation, intestinal obstruction, duodenal web, biliary atresia, foregut duplication cyst
Central nervous system (CNS)	8	Microcephaly, hydrocephalus, Bell's palsy, CNS arterial malformations, abnormal pituitary, absent septum pellucidum/corpus callosum, hyperreflexia, neural tube defection, Arnold-Chiari malformation, Moyamoya, single ventricle

Approximately half of children with FA have congenital skeletal anomalies, frequently of the thumb (hypoplastic, duplicated, or absent) and the radius of the forearm (smaller or absent) [8-10]. However, in the most severe cases, developmental abnormalities simultaneously affect many organ systems and involve the central nervous system and the gastrointestinal system [11-13]. FA patients may present with vertebral anomalies, anal atresia, cardiac abnormalities, tracheo-esophageal fistula, renal anomalies, and sometimes hydrocephalus. More than 50% of patients with FA have short stature associated with deficiencies in growth hormone production and hyperthyroidism (Figure 1.1) [14, 15]. Other endocrine dysfunctions are also associated with FA including abnormal blood glucose regulation, osteoporosis, insulin metabolism

and diabetes [14-16].

The congenital malformations observed in FA probably represent the final outcome of inappropriate cell death during embryogenesis [17]. Evidence has shown that this abnormal apoptosis is likely related to increased inactivation of p53 due to the inability of Fanconi cells to repair DNA damage that occurs during development [18].

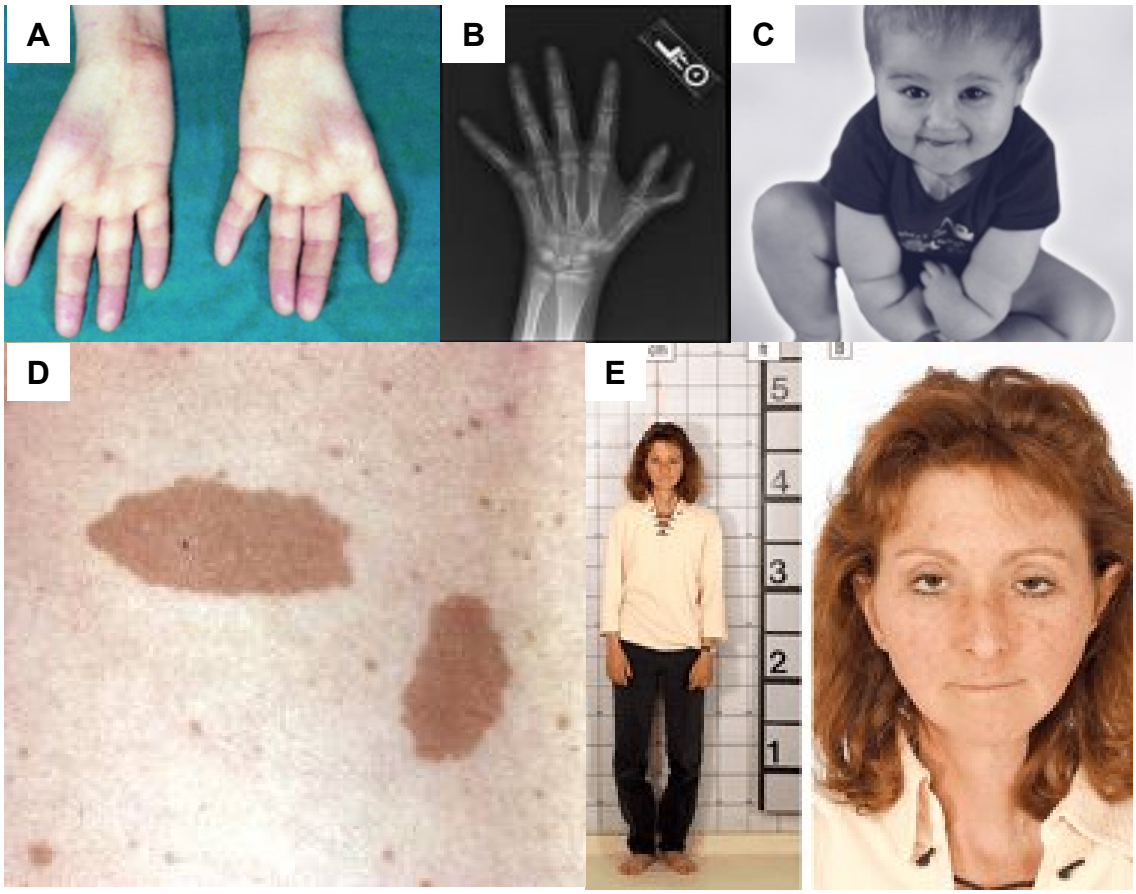

Figure 1.1: Congenital abnormalities of FA. (A) Absence of thumb. (B) Duplication of thumb. (C) Radial deviation of hand. (D) Café-au-lait spots and hyperpigmentation. (E) Short stature and microphthalmia. Images adapted from internet.

1.1.2.2 Hematological manifestations

A common clinical manifestation in most patients with FA is life-threatening hematologic abnormalities that occur at a median age of 7 years (range: birth to 41 years) [7, 19-21]. The bone marrow dysfunction in these patients may arise from a deficiency in

the pool of hematopoietic stem cells [7, 21]. The FA fetus may be exposed to a stochastic source of DNA damage in utero such as ionizing, ultraviolet radiation or ROS that drives both developmental abnormalities and the depletion of embryonic hematopoietic reserves. The depletion of hematopoietic reserves that occurs during the development of these patients may subsequently promote a rapid progression to aplastic anemia (AA) within the first decade of life, this condition may then be followed by thrombocytopenia, leucopenia, and finally progression to full-blown pancytopenia [21]. Notably, some patients present with myelodysplastic syndrome (MDS) or acute myeloid leukemia (AML) without prior diagnosis of AA. The risk of AML occurrence is approximately 800-fold higher than that of the general population, with a median age of onset of 14 years. The cumulative incidence of MDS by age 40 years was 33% [7].

Recent reports have revealed that the most frequent chromosomal abnormalities in FA patients with MDS or AML are translocations of chromosome 1q, monosomy 7, and gains of 3q [21-23]. Deletions of 5q or 11q, rearrangements of 6p, and gains of chromosomes 8 and 21 have also been noted by different groups [23]. In addition, AML in FA patients rarely involves the chromosomal rearrangements (e.g., t(15;17), t(8;21), and inv(16) or t(16;16)) observed in non-FA patients with AML [15, 16, 24, 25]. The exact cause of these hematopoietic defects is unclear, although increasing evidence suggests an underlying intolerance of FA hematopoietic cells to oxidative stress [16].

1.1.2.3 Neurological defects

Distinct neuropathology has been reported in approximately 8% of FA patients. In many cases, predominant ventricular defects were noted together with associated anomalies including unilateral microphthalmia and cell migration defects [26]. Other neurological abnormalities associated with FA include microcephaly, hydrocephalus, Bell's palsy, CNS, arterial malformations, abnormal pituitary, absent septum

pellucidum/corpus callosum, hyperreflexia, neural tube defects and Arnold-Chiari malformation [26]. Currently, no data are available that correlate specific neurological lesions with particular FA complementation groups.

1.1.2.4 Neoplasia predisposition

FA patients display a significant predisposition to neoplasia, both to leukemia and to solid tumor formation. In fact, FA patients have a much greater risk of developing leukemia acute myeloid leukemia (AML) than people without FA, with a median age of 14 years at diagnosis [21, 27, 28]. Up to 34% of patients with FA have MDS [21, 27, 28], and a significant proportion develop leukemia. Hematologic malignancy is the major cause of early death in FA patients, who have a median estimated survival of 23 years and 81% risk of death by the age of 40 years.

In addition to the extraordinarily high frequency of leukemia in FA patients [21], the high incidence of non-hematologic malignancies in these patients is especially striking and represents the predominant clinical problem for adult FA patients. Improved management of young FA patients, which includes new transplant protocols, and more rigorous diagnostic testing in adults has resulted in improved probability that these patients will survive to an age at which the incidence of solid tumors begins to increase. Most of the non-hematologic tumors in FA patients are squamous cell carcinomas (SCC), especially of the head and neck, esophagus and anogenital regions [7, 29]. FA patients have a 500- to 700-fold higher incidence of head and neck SCC than the general population and a 14% cumulative incidence of head and neck SCC by the age of 40 years [30]. The cited study suggests that FA is associated with increased susceptibility to HPV-induced carcinogenesis and that SCC in FA patients is probably associated with the inactivation of p53 by HPV-associated oncoproteins rather than by direct mutagenesis. However, published studies have yielded conflicting results [31-33].

1.1.3 Diagnosis and treatment of FA

1.1.3.1 Diagnosis

FA patients may present with various congenital abnormalities and often reveal the presence of the disease prior to age 12; however, in rare cases, no symptoms are present until adulthood. It is recommended that all patients exhibiting any congenital malformation known to be associated with FA or AA, and all patients with MDS with complex cytogenetic abnormalities be tested for FA[8]. Any patient who develops SCC of the head and neck, gastrointestinal or gynecologic system at an early age should be tested for FA. Testing for FA is essential for patients with AA and prior to stem cell transplantation because standard chemotherapy and radiation protocols may prove toxic to FA patients.

The definitive test for FA is the assessment of cellular hypersensitivity to DNA interstrand crosslinking agents (ICLs) such as diepoxybutane (DEB) and mitomycin C (MMC) [34]. Exposure of FA cells to these agents results in high levels of chromosomal aberrations, particularly chromosomal breaks and radial formations (Figure 1.2). The level of chromosome breakage in primary lymphocytes from a patient sample is compared to that in cells from known FA patients and from normal control subjects. These tests can also be performed prenatally on cells from chorionic villi or from the amniotic fluid. The test yields occasional false positives because the presence of other genetic disorders such as Nijmegen breakage syndrome and Roberts syndrome also results in the production of aberrant chromosomes upon exposure to these DNA ICLs [35].

FA cells contain an increased proportion of cells in G2/M phase both before and after treatment with DNA ICLs [35]. This can be used as an alternative diagnostic method for FA. Subtyping the complementation group of FA has become increasingly important [36,

[37] because the clinical course of the disease varies depending on the subtype. FA patients with FANCA tend to display a milder disease phenotype with later onset of bone marrow failure, whereas patients with subtypes FANCC and FANCG tend to have more severe disease and require earlier intervention. Patients with FANCD1 show predisposition to leukemia and solid tumors in early childhood [38]. In this test, FA cellular phenotypes such as the presence of chromosomal aberrations and hypersensitivity to DNA ICLs are corrected by the transduction of cDNA from the appropriate FA complementation group. The subtyping test helps confirm the diagnosis and provides a basis for determining the optimal care for each patient.

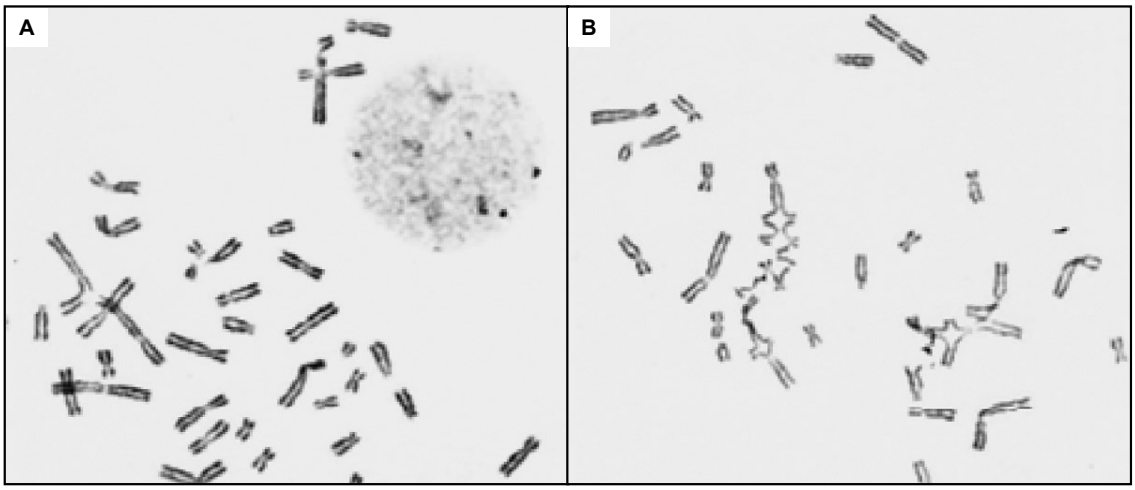

Figure 1.2: Chromatid damage after treatment of cells from FA patients with ICLs. (A) FA lymphocytes showing spontaneous chromatid aberrations. (B) FA lymphocytes showing multiple complex chromatid exchange. Adapted from Auerbach, A.D., et al. Pediatrics, 1981.

1.1.3.2 Treatment

Bone marrow failure (BMF) BMF is the most common clinical manifestation in FA patients and typically develops during the first decade of life [39]. Approximately

half of FA patients respond well to androgens, which stimulate the production of red blood cells and platelets and sometimes increase white cell production [27]. Long-term androgen use has significant side effects including hirsutism and increased liver tumor incidence. Although this treatment may be effective for many years, in most patients the disease becomes refractory to androgen therapy. For these patients, hematopoietic stem-cell transplantation (HCT) may be considered and in this context, the use of androgens should be avoided because androgen treatment can adversely affect the ultimate success of a transplant. Hematopoietic growth factors (e.g., G-CSF and GM-CSF, which stimulate the production of white blood cells) are also effective in some FA patients [40].

At the present time, HCT remains the primary treatment for bone marrow failure in FA. This treatment is associated with numerous risks, and these risks are highly compounded in FA patients because of the underlying DNA repair defect. FA patients are extremely sensitive to the radiation and chemotherapy used in the transplantation procedure, and the survivors often experience multiple complications that are not routine for other transplants such as marked increased organ (pulmonary and renal) toxicity, graft-versus host disease (GVHD), immune injury, sterility, and endocrinopathies [41-45]. Histocompatible sibling donor transplants generally result in the best outcomes for FA patients [39, 41]. Because most FA patients do not have histocompatible siblings, some families have turned to preimplantation genetic diagnosis (PGD) [46]. While HCT is highly successful in extending the life expectancy of FA patients, managing the long-term complications of HCT is a significant concern.

Management of cancers With successful prolongation of the survival of FA patients after HCT, the treatment of cancers becomes more significant. Because FA patients are highly sensitive to chemotherapy and radiotherapy, treatment strategies for

FA-related cancers must differ from those for non-FA cancers. Prevention and close surveillance of cancer occurrence become more important; for instance, frequent dental evaluations and gynecologic exams of FA patients should be conducted to identify related early SCCs [47, 48]. Some FA complementation groups correlate with specific cancer patterns; for example, patients with the FANCD1 subtype of the disease have earlier onset and increased incidence of a variety of tumors (Wilms tumor, neuroblastoma), and patients from subtype J, N, and O families are highly predisposed to breast, ovarian, and pancreatic cancers [38, 49, 50]. Specific close surveillance and preventive treatment must be provided for patients of these FA subtypes.

Other potential therapies FA is an ideal candidate for some potential therapies. Over 98% of FA cases are associated with identified FA genes (from FANCA to FANCP). Subtyping FA allows identification of the mutant FA gene(s) present in an individual and the generation of related retroviral or lentiviral vectors carrying the wild-type cDNA, which can be used in gene therapy. However, technical obstacles such as insufficient HSCs for ex vivo transduction, potentially inserted mutagenesis and expansion of malignant clones often must be overcome prior to successful gene therapy [51]. Because ROS play a critical role in endogenous DNA damage, especially in FA, antioxidants are potential therapeutic compounds for FA treatment, with the limitation that apoptosis is often induced by the excessive use of antioxidants [52-54]. Some small molecule inhibitors, such as inhibitors of CHK1, DNA-PK, and p38 MAP kinase, may be potential therapeutic molecules with the limitation that they might enhance tumorigenesis or immune suppression [55]

1.2 Molecular basis of FA

1.2.1 FA genes

To date, 19 complementation groups for FA have been identified. All of these

groups correspond to one of the following cloned genes: *FANCA, FANCB, FANCC, FANCD1/BRCA2, FANCD2, FANCE, FANCF, FANCG, FANCI, FANCJ/BRIP1/BACH1, FANCL/PHF9, FANCM/HEF, FANCN/PALB2, FANCO/RAD51C, FANCP/SLX4*, *FANCQ/XPF4/ERCC4, FANCR/RAD51* and *FANCS/BRCA1, FANCT/*UBE2T (Table 1.2), [56-74]. The biallelic disruption of any of these genes results in human disease, as outlined in Table 1.2. *FANCA, FANCC* and *FANCG* are the three most commonly defective genes in FA and mutations of these genes can be detected in approximately 85% of FA patients. *FANCD1, FANCD2, FANCE, FANCF* and *FANCL* account for 10%, while the remaining FA genes, *FANCB, FANCI, FANCJ, FANCM, FANCN, FANCO, FANCP, FANCQ,FANCR* and *FANCS* represent less than 5%. Some individuals with FA do not appear to have mutations in these 18 genes, indicating the possibility that novel FA genes exist [75]. *FANCB* is unusual among the FA genes because it is on the X chromosome, whereas the other FA genes are located on autosomes.

FA patients with mutations in any of the FA genes present characteristic clinical features, although to various degrees, and a common cellular phenotype. This indicates that the 18 known FA genes function in the same DNA repair pathway, termed the FA pathway [76]. The discovery that the *FANCD1* gene is identical to the breast cancer susceptibility gene *BRCA2* [67] and *BRCA1* corresponds to *FANCS* [77], connects the FA pathway and breast cancer. Identification of *FANCN* as *PALB2*, a partner and localizer of *BRCA2* [78] [79], *FANCJ* as *BRIP1*, the *BRCA1*-interacting helicase1 [71, 80], and recently, *FANCO* as *RAD51C*, a breast and ovarian susceptibility gene [81, 82], further emphasize the close association of breast and ovarian tumor suppressive genes with FA and the association of both diseases with DNA repair mechanisms.

Table 1.2: FA complementation groups.

Gene (alias)	Mutation frequency	Locus	Protein MW (kDa)	Protein function
FANCA	~66%	16q24.3	163	Core complex
FANCB	~2%	Xp22.31	95	Core complex
FANCC	~10%	9q22.3	63	Core complex
FANCD1/BRCA2	~2%	13q12.3	380	Recruits RAD51 and promotes HR repair
FANCD2	~3%	3p25.3	162	Monoubiquitinated. Recruits FAN1, FANCP to chromatin, exonuclease activity
FANCE	~2%	6p21.3	60	Interact with FANCD2
FANCF	~2%	11p15	42	Core complex
FANCG/XRCC9	~10%	9p13	70	Core complex
FANCI	<2%	15q25-26	150	Monoubiquitinated. Forms heterodimer with FANCD2
FANCJ/BRIP1	<2%	17q22.3	150	Interacts with BRCA1. DNA helicase, ATPase
FANCL/PHF9	Rare	2p16.1	43	E3 ubiquitin ligase
FANCM	Rare	14q21.3	250	Helicase/translocase
FANCN/PALB2	Rare	16p12.1	130	Mediates interaction between BRCA1 and BRCA2 during HR
FANCO/RAD51C	Rare	17q22	43	Promotes HR, RAD51 paralogue
FANCP/SLX4	Rare	16p13.3	200	Holliday junction resolvase
FANCQ/XPF4/ERCC4	Rare	16p13.12	105	DNA repair endonuclease
FANCR/RAD51	Rare	15q15.1	37	Activation of HR and DSB repair
FANCS/BRCA1	Rare	17q21	207	Multifunction in DNA damage repair
FANCT/UBE2T	Rare	1q32.1	23	Ubiquitin-Conjugating Enzyme E2T

1.2.2 FA pathway

In normal cells, the FA pathway is activated during the S phase of the cell cycle and in the presence of DNA damage. In the FA pathway, FA proteins interact to form three specific complexes. Eight upstream FA proteins (FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCL, and FANCM) form complex I, termed the FA core complex [68, 73, 83-91]. The primary function of the FA core complex is to monoubiquitinate the two FA proteins FANCD2 and FANCI, components of complex II (the ID complex), through the activity of the FANCL E3 ubiquitin ligase (Figure 1.3) [68, 92-94]. Several other FA-associated proteins are essential for the function of the core complex, including FA-associated 24-kDa protein (FAAP24)[90], FAAP100[91], FANCM-associated histone fold protein 1 (MHF1), MHF2 [95], hairy enhancer of split 1

(HES1), and the recently identified FAAP20 [96]. The disease-causing mutations in these FA-associated proteins have yet to be identified.

Homologous recombination (HR)-based repair is the major DNA repair pathway regulated by the FA proteins. In response to DNA damage, an ATR-mediated signal activates the FA core complex and monoubiquitination of the ID heterodimer. The ubiquitin-tagged ID complex move to chromatin, where it assembles nuclear DNA-repair foci. In these foci, the ID complex functionally associates with the downstream FA proteins (complex III) FANCD1, FANCN, FANCO, FANCJ and with some key HR factors such as BRCA1 and RAD51 [81, 97]. In addition to HR, the FA pathway also mediates nucleotide incision repair (NER) and translesion synthesis (TLS). FAN1 nuclease and FANCP are recruited with ID complex and participate in the nucleolytic incision of cross-linked DNA. The TLS polymerase is essential for TLS at the step following the incision of ICL. The FA network also includes other regulatory proteins such as ubiquitin-specific peptidase 1 (USP1) and the USP1-associated protein UAF1; these function as deubiquitination enzymes of the ID complex and are required for completion of the FA pathway [98, 99].

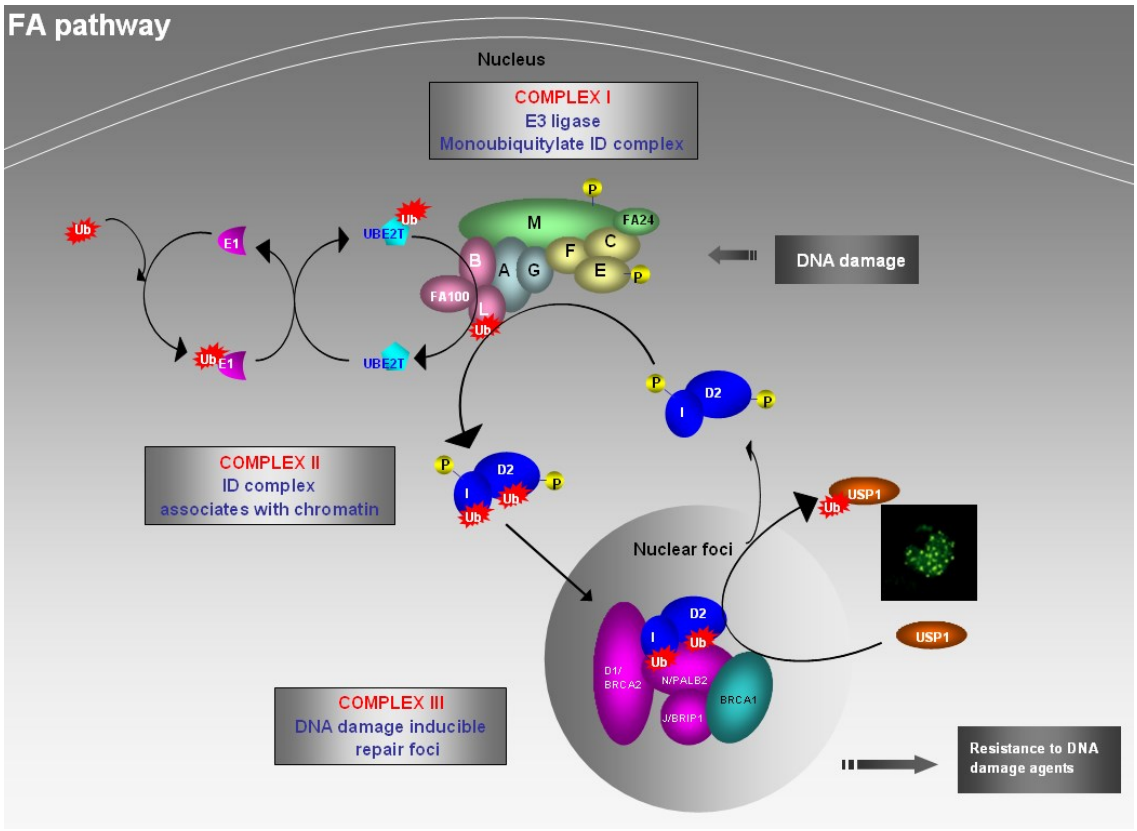

Figure 1.3: Canonical FA pathway. DNA damage activates the FA core complex (A, B, C, E, F, G, L, and M). The FA core complex then functions as an E3 ubiquitin ligase and monoubiquitylate the ID complex. The monoubiquitylate ID complex targets to chromatin, forms nuclear foci, and associates with other DNA repair proteins to repair DNA damage. The USP1 and UAF1 protein complex is required to deubiquitylate the ID complex and complete the FA pathway. Fengfei Huang, 2014.

1.3 Apoptosis and FA

Apoptosis is the process of programmed cell death (PCD) that may lead to characteristic cell changes (morphology) and cell death. Defective apoptotic processes have been implicated in a wide variety of diseases such as cancer. In addition, apoptosis also confers advantages during tissue development. For example, the separation of fingers and toes in a developing human embryo occurs because cells between the digits undergo apoptosis.

The environmental stress such as glucocorticoids, heat, radiation, nutrient deprivation [100], viral infection can all trigger the intracellular apoptotic signals. Two main methods of regulation have been identified: targeting mitochondria functionality, or directly transducing the signal via adaptor proteins such as TNF-induced (tumour necrosis factor) model and the Fas-Fas ligand-mediated model. The formation of mitochondrial apoptosis-induced channel (MAC), release of small mitochondria derived activator of caspases (SMACs), Cytochrome c into the cytosol are essential process in mitochondrial regulation. Both TNF path and Fas path involve the formation of death domain (DD) and either indirect activate transcription factors or initiate caspase signaling pathway.

Significant evidence supports abnormal apoptosis of HSPCs [101, 102] occurs in the pathogenesis of BMF and leukemia progression in FA. Increased apoptosis due to higher level of the death receptor Fas (CD95) in CD34+ cells was first reported in children with FA [102]. Subsequently, enhanced TNF-α-induced apoptosis is found in FANCC-deficient cells and FANCC could modulates apoptotic responses to tumor necrosis factor-alpha (TNF-α) and Fas ligand [103]. Elevated expression of TNF-related apoptosis-inducing ligand (TRAIL) at the bone marrow level has also been found in FA, which may also implicate in the pathogenesis of FA[104] and MDS[105]. In addition, FANCD2 was shown to be a target for caspase 3 during DNA damage-induced apoptosis. These data indicate that abnormal apoptosis in the condition of FA may at least partially explain the BMF and leukemia in FA patients.

1.4 FANCC protein

FANCC gene deficiency is one of the most frequent mutations in FA patients. The gene codes for a 63-kDa protein, FANCC protein, which was the first FA protein to be identified. In addition to its critical role as part of the core complex needed to confer

cellular resistance to DNA damage, FANCC also participates widely in other non-repair pathways of oxidative metabolism, cell cycle progression and apoptosis.

1.4.1 Cellular localization of the FANCC protein

In the presence of DNA damage, the core complex is formed by the direct interaction of FANCA with FANCI, FANCB, FANCG and FANCM. FANCF interacts directly with FANCL, FANCG, and with FANCC-FANCE heterodimer. FANCM is essential for the loading of complex on chromatin (Figure 1.4) [106, 107]. Although in the canonical FA pathway the core complex proteins cooperate and function in cell nucleus, some of these proteins can be detected in the cytoplasm. For instance, FANCC protein can be found in the nucleus but is principally located in the cytoplasm [108]. The nuclear accumulation of both FANCC and FANCE is mutually dependent and is crucial for the function of the FA core complex [109]. The molecular chaperone glucose-related protein 94 (GRP94) has been shown to directly bind FANCC and to be involved in the regulation of the levels of the proteins in each cellular compartment [110]. In addition to the formation of the FANCC/FANCE subcomplex, other subcomplexes, including FANCA/FANCG [87, 89] and FANCB/FANCL [69], can be found in the cytoplasm, suggesting that FA proteins have cellular functions in addition to DNA damage repair (Figure 1.4).

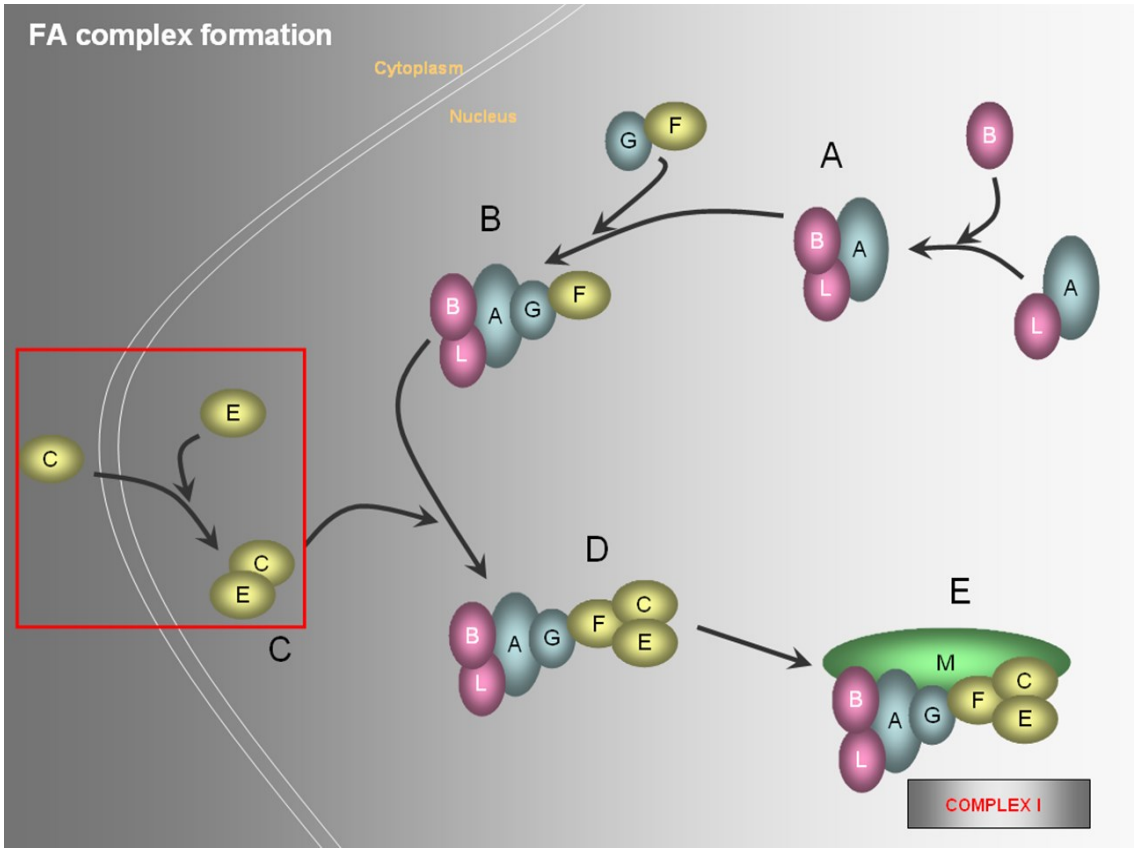

Figure 1.4: FA core complex formation. (A) FANCA and FANCL first recruit FANCB to the nucleus. (B) The FANCF-FANCG heterodimer then binds to the FANCA-FANCB-FANCL subcomplex. (C) The accumulation of FANCC in the nucleus requires FANCE. (D) The FANCC-FANCE heterodimer binds to the free nuclear complex through the interaction of FANCC with FANCF. (E) The nuclear complex is then loaded on chromatin through its interaction with FANCM. Adapted by Fengfei Huang from internet, 2014.

1.4.2 Posttranslational modification of FANCC protein

Posttranslational modification is critical in triggering the activity of FA proteins. FA proteins undergo multiple posttranslational modifications; some examples are the well-characterized monoubiquitination of FANCD2 and FANCI [111] and the phosphorylation of FANCA, FANCE, FANCG, FANCD2, FANCI, and FANCM [112-117].

Unlike most other known FA proteins, FANCC function is regulated through a caspase-mediated proteolytic process [118]. Recently FANCD2 has also been shown to be regulated through by caspase3 during apoptosis induced by DNA damage [119].

During apoptosis, FANCC undergoes proteolytic modification by a caspase that produces a predominant 47-kDa ubiquitinated protein fragment by cleavage at position 306 (LETD ⇓G) (Figure 1.5). This modification is not required for FANCC to function in DNA damage signaling but inhibits its function as a suppressor of apoptosis [118]. The exact caspase responsible for FANCC cleavage has yet to be determined. Further studies are also required to investigate whether the C-terminal FANCC fragment has a proapoptotic function.

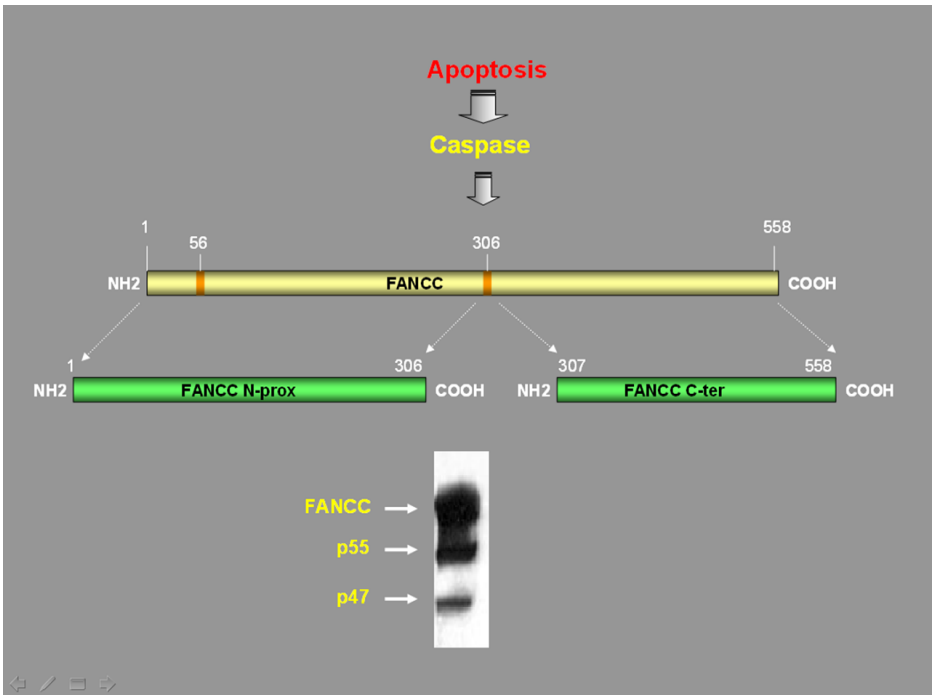

Figure 1.5: Caspase-mediated proteolytic modification of FANCC. FANCC is regulated through proteolytic processing by a caspase during apoptosis. Cleavage of the protein at position 56 (^{53}KEMD ⇓ S^{57}) and at position 306 (^{303}LETD⇓ G^{307}) gives rise to a 55-kDa protein fragment and to the C-terminal 47-kDa FANCC fragment. Adapted from Brodeur, I., et al..J Biol Chem.

1.4.3 Developmental expression of the *FANCC* gene

The congenital abnormalities associated with FA imply that FA genes play important roles in normal development. Murine *FANCC* protein shares 67% amino acid identity with its human counterpart, and the function of the protein is conserved [120]. Analysis of *FANCC* gene expression during murine development provides clues to the function of the FANCC protein and an understanding of the basic defect in the disease. *FANCC* is observed initially in the mesenchyme at 8–10 days gestation, during the later stages of bone development (13–19.5 days) and later in cells of osteogenic and hematopoietic lineage. *FANCC* transcripts are also expressed in cells within the intramembranous cranial and facial bones. *FANCC* mRNA is also present in non-skeletal tissues: brain, whisker follicles, lung, kidney, gut and stomach (Figure 1.6) [121]. The expression pattern of *FANCC* is consistent with the congenital defects observed in FA patients. Interestingly, *FANCC* is highly expressed in rapidly dividing progenitor cells but is downregulated in differentiating cells. This is consistent with the hypothesis that structures with high rates of cell replication are the structures most likely to be affected by mutations in FA genes [122] and implies a possible role for FANCC protein in DNA repair.

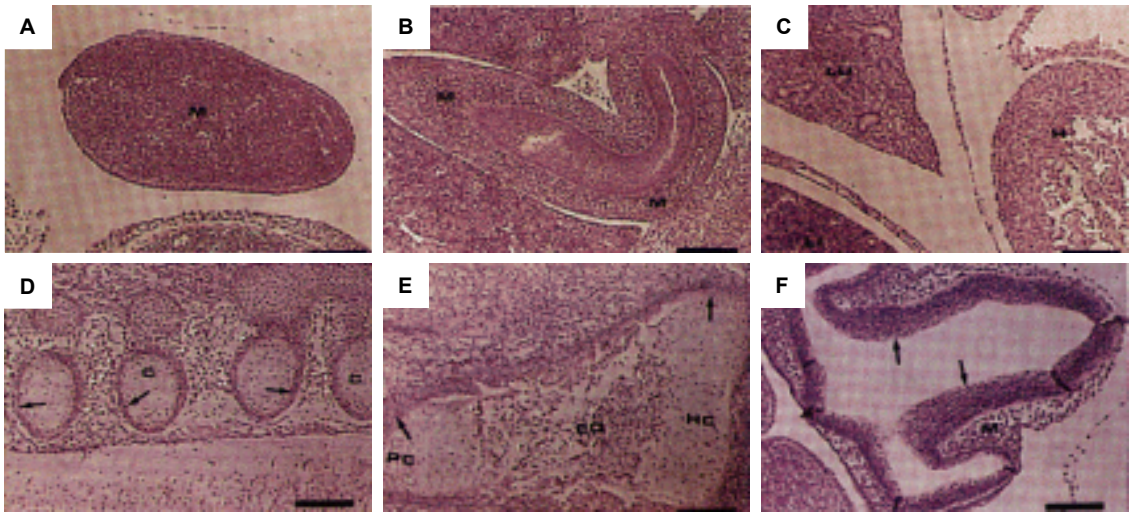

Figure 1.6: *In situ* hybridization analysis of embryonic *FANCC* expression in multiple tissues. (A) Hindlimb bud at 10 days; (B) Gut at 13 days; (C)Lung at 15 days; (D) Vertebrae at 13 days; (E) Femur at 16 days; (F) Developing brain at 9 days [121]. Adapted from Krasnoshtein, F., et al. Hum Mol Genet, 1996.

1.4.4 The role of FANCC in oxidative metabolism

A number of studies have indicated that abnormal oxidative metabolism occurs in FA cells. FA cells show increased ROS levels and hypersensitivity to reactive oxygen species (ROS) (Table 1.3) [123]. The altered redox state of the cells is linked to specific features of the FA cell such as reduced proliferation, reduced growth, and altered cytokine responses; these features contribute to the pathogenesis of bone marrow failure and leukemia progression [124]. Prooxidant states were found in white blood cells and in body fluids from FA patients, which show excessive levels of luminol-dependent chemiluminescence (LDCL), 8-hydroxy-deoxyguanosine (8-OHdG), tumor necrosis factor-α (TNF-α), and reduced glutathione (GSH)/oxidized glutathione imbalance [125-127]. All of these data indicate that FA proteins may function to mediate endogenous oxidative metabolism. For instance, FANCC protein has been shown to interact with redox-related molecules including NADPH cytochrome-P450 reductase (RED) and glutathione S-transferase πI (GSTπI) [128, 129], implying a role of FANCC in attenuating the redox activation of xenobiotics. Mitochondrial dysfunction, including peroxyredoxin 3 (PRDX3) cleavage and decreased peroxidase activity, has been described in FANCC-defective cells [130]. In addition, FANCC-defective lymphoblastoid cells showed acute adenosine triphosphate (ATP) depletion and significant apoptosis after treatment with rhodamine-1,2,3 and doxycycline [131].

Other FA proteins, such as FANCA and FANCG, are also involved in redox

homeostasis regulation as shown by their association with cytochrome P450-related activities and by the presence of distorted mitochondria and decreased peroxidase activity [130, 132, 133]. FANCD2 interacts with ataxia telangiectasia mutated protein (ATM) and forkhead box O3 (FOXO3a) in ROS, and FANCJ has been identified as a repressor of the heme oxygenase-1 gene [134-137]. Together, these data suggest that it might be possible to design chemoprevention protocols to counteract some of the clinical implications of FA.

1.4.5 The role of FANCC in cytokine signaling and apoptosis regulation

Extensive studies have shown that FA mutant cells undergo increased apoptosis or reduced cell growth in response to stimulation by various agents such as ROS inducers, DNA-damaging agents, growth factor withdrawal, and cytokines. This implies that the FA proteins may function in pathways that regulate cell survival [138-141]. FANCC, as a caspase-mediated target, has been reported to prevent apoptosis when overexpressed or when its cleavage is inhibited and thereby to function as a cell survival protein (Table 1.3) [101, 142]. Several clues suggest that altered cytokine regulation plays an important role in the progression of the FA phenotype. For instance, increased TNF-α and induced Il-6 levels have been observed in FA patients, and the cytokine response genes myxovirus A (MxA), IFN response factor 1 (IRF1), p21CIP/WAF, and IFN-stimulated gene factor 3(ISGF3γ) also show high expression in FA mutant cells [143-145]. Consistent results from an FA mouse model show that continuous injection of low doses of IFN-γ in vivo leads to BMF [146, 147], whereas TNF-α treatment leads to the clonal evolution of leukemias [148]. Both human and mouse FA-C mutant cells have been observed to be hypersensitive to TNF-α and IFN-γ, indicating that the cell survival function of FANCC might operate through the modulation of cytokine signaling [103, 142, 149].

Indeed, FANCC was shown to directly interact with the signal transducer and

activator of transcription 1 (STAT1) and subsequently to activate the IFN type II signaling cascade [150], whereas in FA-C cells STAT1 activation is defective. Other cytokines, including IFN-α, granulocyte macrophage colony stimulating factor (GM-CSF) and stem cell factor are involved in inducing the FANCC-STAT1 interaction. An abnormal response to type I IFN was also detected in FA-C cells; this response was related to reduced phosphorylation of the Janus kinases Jak1 and Tyk2, which induces a decrease in CD4-positive cell numbers, possibly resulting in immune defects like those reported in FA patients [151-153]. FANCC may also participate in the protection of cells against TNF-α- and IFN-γ-induced apoptosis through interaction with Hsp70. This interaction also inhibits the activity of IFN-inducible double-stranded RNA (dsRNA) and RNA dependent protein kinase (PKR) [154, 155]. Other FA proteins such as FANCA have also been reported to be involved in cytokine signaling and in the regulation of apoptosis; these proteins have been shown to interact with IKK2 to mediate the cellular response to stressors such as dsRNA and cytokines [156-158].

1.4.6 The role of FANCC in transcription

Another important but less studied role of FANCC is transcription regulation. In the yeast two-hybrid system, FANCC has been shown to directly interact with FA zinc finger (FAZF) protein [159], a transcriptional repressor, and is plausibly hypothesized to interfere with the transcription of critical target genes required for growth suppression in hematopoietic progenitor cells. Another transcriptional repressor identified as a FANCC protein partner is hairy enhancer of split 1 (HES1) [96]. Actually, HES1 was shown to interact directly with several components of the FA core complex. The FA core complex was shown to regulate the transcription of HES1-responsive genes, both positively (*HES1*) and negatively (cyclin-dependent kinase inhibitor $p21^{cip1/waf1}$) [96].

Table 1.3: FANCC multifunctionality through its interacting partners.

Interact partner	Functional class	Specfic function	References
RED	Oxidative metabolism	Electron transfer	Kruyt, Hoshino et al. 1998, Reuter, Medhurst et al. 2003
GSTP1	Oxidative metabolism	Cytosolic Detoxifying enzyme	Cumming, Lightfoot et al. 2001, Reuter, Medhurst et al. 2003
STAT1	Cell signaling	Cytokine response	Pang, Fagerlie et al. 2000
cdc2	Cell cycle	Serine/threonine kinase	Kupfer, Yamashita et al. 1997, Reuter, Medhurst et al. 2003
FAZF	Transcription	Transcriptional repressor	Hoatlin, Zhi et al. 1999, Reuter, Medhurst et al. 2003
Hsp70	Transcription	Stress-induced chaperone	Pang, Keeble et al. 2001, Reuter, Medhurst et al. 2003
GRP94	Transcription	Stress-induced chaperone	Hoshino, Wang et al. 1998, Reuter, Medhurst et al. 2003

1.4.7 UNC5A, the novel protein partner of FANCC

Intensive studies of FA protein focused on DNA repair have described a canonical FA pathway that could explain some features of the FA phenotype. However, considerable evidence suggests that FA proteins may have other functions in addition to their roles in DNA damage signaling. As a protein predominantly located in the cytoplasm, FANCC assumes multifunctional roles, especially that of an anti-apoptotic regulator.

To better understand the biological functions of FA proteins, a yeast two-hybrid screen was conducted using FANCC proteolytic fragments. One of the clones obtained was identified as UNC5A. UNC5A belongs to the UNC5 family of proteins (human UNC5A-D and rodent UNCH1-4), which are receptors of the axon guidance molecule, Netrin-1. The Netrin-1 signaling pathway primarily functions to provide migrational cues in the developing central nervous system (CNS). Recently, Netrin-1 has been shown to regulate diverse processes such as cell survival, tissue development, and guidance cues during angiogenesis in a number of non-neuronal tissues. The crosstalk between FA and the Netrin-1 signaling pathway might explain some FA phenotypes, in particular the occurrence of abnormal apoptosis, defects in hematopoiesis and

congenital malformations.

1.5 Netrin-1 signaling pathway

In 1990, three *Caenorhabditis elegans* genes, unc5, unc6, and unc40, were found to be involved in pioneer axon guidance and cell migration. This marked a significant advancement in neural development research [160]. The unc-5, unc-6, and unc-40 genes encode proteins that guide the circumferential migration of pioneer axons and mesodermal cells on the epidermis in *C. elegans*. In 1994, the vertebrate homologue of unc6, Netrin-1 (named after Sanskrit 'netr', meaning 'guide'), was identified and shown to have a similar function as a chemotropic guidance cue for migrating cells and axons [161]. Later, the chemoattractive guidance receptor for Netrin-1, Deleted in Colorectal Cancer (DCC), and its chemorepulsive receptor, uncoordinated-5 (Unc5) family, were identified as the mammalian orthologues of unc40 and unc-5 [162, 163]. Studies of the Netrin signal pathway have been conducted in a wide range of animal species, including invertebrates such as *Caenorhabditis elegans* and *Drosophila melanogaster*, non-mammalian vertebrates such as *Xenopus laevis*, and mammals including rats, mice and humans. In addition to functioning as guidance cues in the CNS, Netrin-1 and its receptors have also been found to play key roles during tissue morphogenesis, angiogenesis and tumor development outside of the nervous system.

1.5.1 The Netrin protein family

Netrins are a family of extracellular proteins. In mammals, three secreted Netrins, Netrins 1, 3 and 4, and two membrane-tethered glycophosphatidylinositol (GPI)-linked Netrins, Netrins G1 and G2, have been identified. All Netrins belong to a superfamily of laminin-related proteins [164]. The N-terminal domains of Netrins contain regions that are highly homologous to domains V and VI of laminins; the N-terminal regions of Netrin-1, 2 and 3 show most similarity to sequences found in the laminin-γ1 chain [161,

[165], whereas the N-terminal regions of Netrins G1, G2 and 4 are most similar to a portion of the laminin-β1 chain [166-168]. Evidence from studies of the structure of Netrins demonstrates that domains VI and V are binding sites for Netrin receptors; domains V1 and V3 are required for unc40-mediated attraction, while domains V1, V2, and V3 are required for Unc5 receptor-mediated responses [169, 170].

Of the secreted Netrins, Netrin-1 expression has been best characterized in all bilaterally symmetrical animals studied so far. Netrin-1 can act as a short-range or a long-range guidance cue. 'Short-range' refers to a role of Netrin-1 in which its guidance occurs either close to the secreting cell or attached to its surface to guide axonal growth cones towards their intermediate or final targets [171]. 'Long-range' guidance, in contrast, is achieved through the establishment of a gradient of Netrin-1 within the extracellular environment and functions at a distance from the cell secreting the factor. In the embryonic nervous system, Netrin-1 secreted by the floor plate cells at the ventral midline of the embryonic neural tube functions as a long-range guidance cue [172], whereas in the adult mammalian CNS, Netrin-1 is expressed by oligodendrocytes, the myelinating cells of the CNS, and mediates cell-to-cell contacts. Netrin-1 is also expressed in other brain regions including the visual system [173-176], olfactory system [174, 177], forebrain [178], cerebellum, forebrain and spinal cord, where it shows a highly conserved function in directing cell and axon migration[173-179]. In addition, Netrin-1 is also highly expressed in various non-neuronal tissues, including the developing heart, lung, pancreas, intestine and mammary gland, where it plays a critical role in tissue morphogenesis, vascular development and tumorigenesis [180], [181].

1.5.2 Netrin receptors and their biological functions

The function of Netrins in chemotropic responses relies on activating and adhesive mechanisms that occur through its binding a number of different receptors including

DCC, the DCC paralogue neogenin, the UNC-5 homologues UNC5A-D, Down syndrome cell adhesion molecule (DSCAM), and the Netrin G ligands (NGLs) NGL-1 and NGL-2. The Netrin receptors described thus far are all single-pass type I transmembrane proteins and members of the immunoglobulin superfamily. The binding of Netrin-1 to its receptors alters the architecture of the cytoskeleton and activates intracellular signal transduction pathways [171]. The specific function of Netrin-1 depends on which receptor is recruited.

Extensive studies have indicated that Netrin-1 is a bifunctional axonal guidance cue and a regulator of neuronal migration regulator in vertebrates, where it mediates chemoattraction or chemorepellence [182]. During the development of the spinal cord, Netrin-1 is secreted by floor plate cells and forms a gradient emanating from the ventral midline. The Netrin-1 gradient acts as a positive guidance cue for the migration of some cells, including spinal commissural axons and dopaminergic neurons (Figure 1.7) [172, 183], and as a repellent guidance cue for other types of cells such as migrating oligodendrocyte precursor cells in the spinal cord, axons of trochlear motoneurons in the brainstem and cerebellar granule neurons [184]. DCC is required for the attractive effect of Netrin-1, while Unc5C is required for the chemorepellent effect [185-187]. Within the mature CNS, Netrin-1 has also been shown to direct the migration of adult neural stem cells to sites of injury [188]. Recent work suggests that Netrins play a role in synaptogenesis between the *C. elegans* AIY interneuron and its DA9 motorneuron. In the head region of the animal, UNC-6 is secreted by glial sheath cells and promotes presynaptic assembly through the DCC/UNC-40 receptor on AIY [189, 190], whereas in the tail UNC-6 secreted by the ventral muscles prevents the mislocalization of presynaptic components to the DA9 motor neuron through the UNC-5 receptor [191, 192].

Netrins and their receptors are also expressed and play key roles in development

outside the nervous system. In mammary gland morphogenesis, Netrin-1 secreted by luminal cells binds to the neogenin receptor expressed by cap cells, stabilizing the adhesive interaction of these cell layers in the developing bud [193]. During the morphogenesis of the lung and the development of the bronchial tree, epithelial stalk cells secrete Netrin-1 and Netrin-4 into the surrounding basal lamina, where they inhibit inappropriate proximal branching and bud formation [180]. During angiogenesis, Netrin-1 inhibits vascular branching through Unc5B secreted by endothelial tip cells; it also promotes the migration and proliferation of vascular endothelial cells via a DCC-dependent mechanism (Figure 1.7) [194, 195].

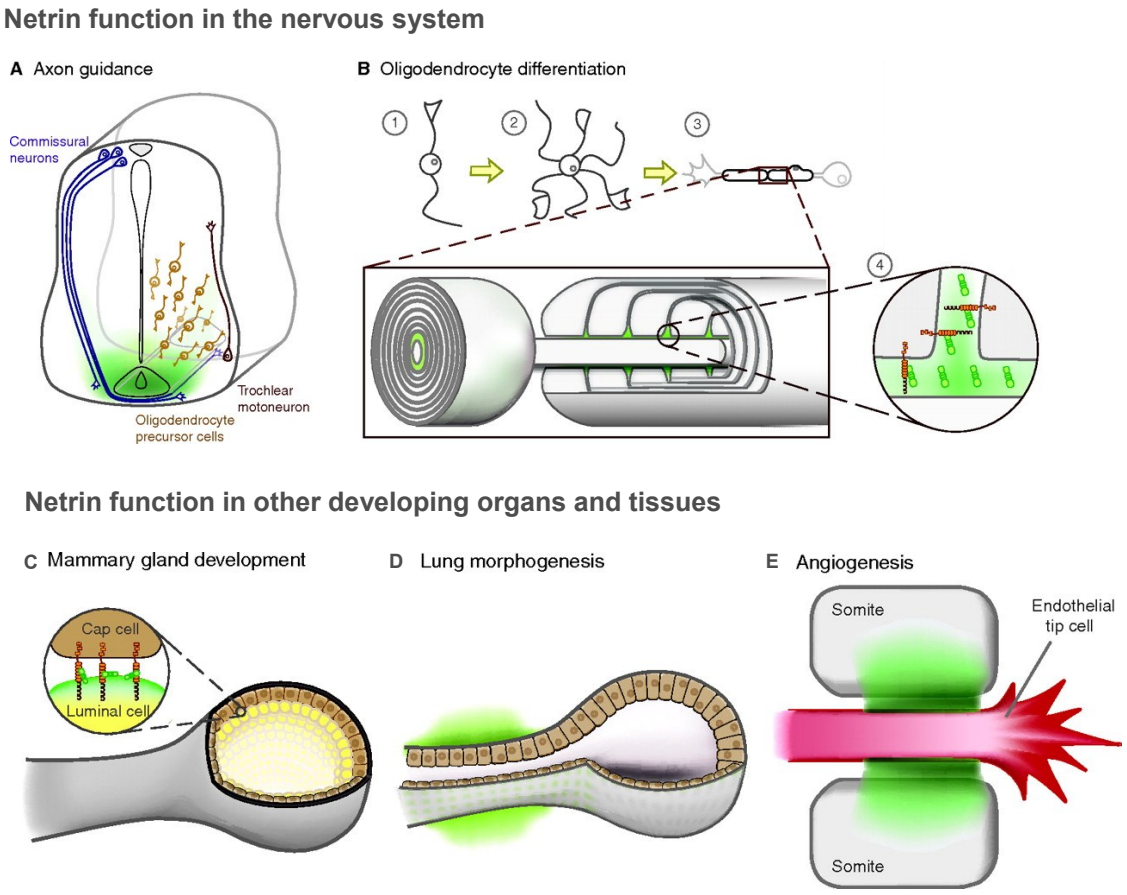

Figure 1.7: Netrin function in nervous system and other developing organs and tissues. (A) Within the developing spinal cord, Netrin 1 (green) secreted by floor plate cells forms a gradient emanating from the ventral midline. (B) Netrin 1 influences

oligodendrocytes at several stages of their differentiation. (C) In mammary gland morphogenesis, Netrin1 (green) is expressed by luminal cells and binds to neogenin (orange) expressed by the cap cells. (D) During lung morphogenesis, epithelial stalk cells secrete Netrin 1 to keep the appropriate proximal branching and bud formation. (E) During angiogenesis, somites secrete Netrin (green) that inhibits vascular branching. Adapted from Karen Lai Wing Sun et al. Development 2011;138:2153-2169.

Netrin-1 and its receptors play an important role in tumorigenesis. It has been proposed that DCC and Unc5 are 'dependence receptors' that, by binding Netrin-1, send a survival signal and that in the absence of Netrin-1 these receptors send signals that promote apoptosis (Figure 1.8) [196]. This pro-apoptotic activity requires caspase cleavage of the receptor's intracellular domain.

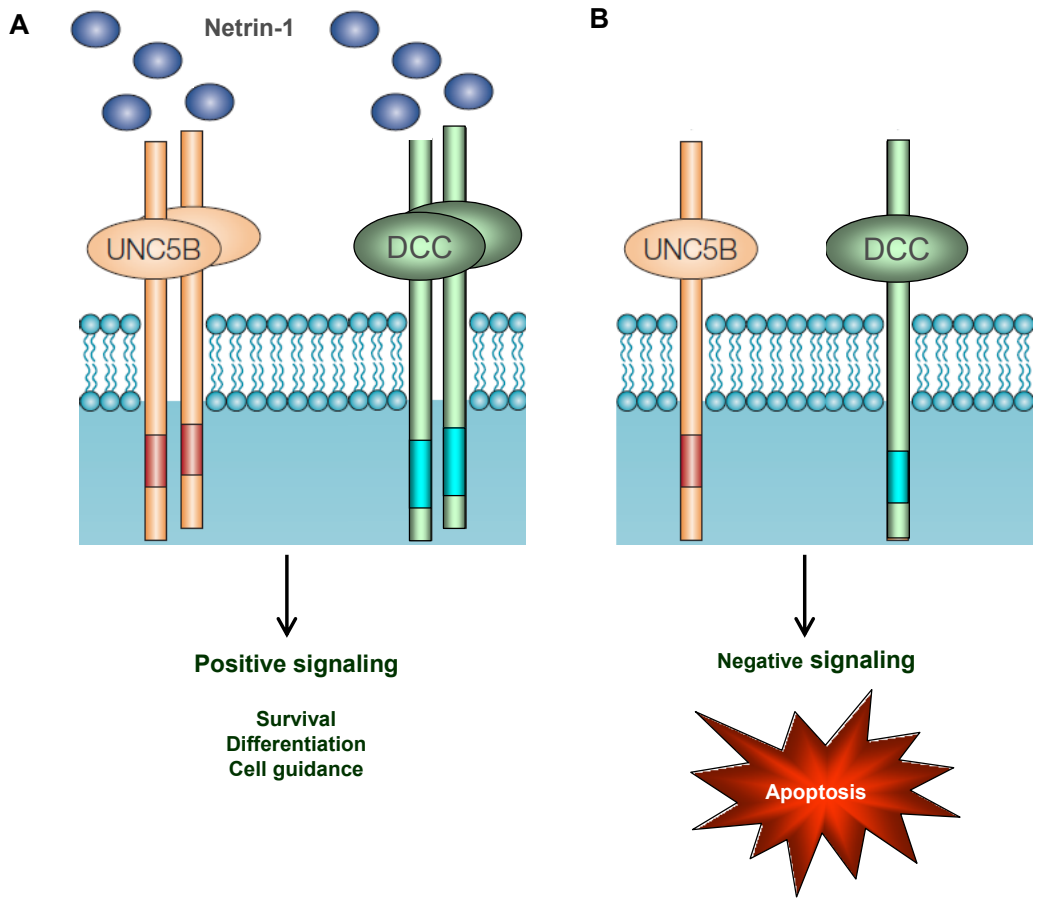

Figure 1.8: DCC and UNC5 belong to the dependence receptor family. (A) A positive signal for migration, proliferation and cell guidance occurs in the presence of the ligand; this signal is associated with dimerization/multimerization of the receptors. (B) A negative signal associated with monomerization of the receptors occurs when the receptors are disengaged from their ligand, inducing apoptosis. Adapted from Arakawa, H., et al. Nat Rev Cancer, 2004.

1.6 UNC5 protein family

UNC5 is a member of the Netrin-1 transmembrane receptor family, which includes the four homologues UNC5A, UNC5B, UNC5C and UNC5D (in humans, these are also called UNC5H1, UNC5H2, UNC5H3 and UNC5H4). As the receptors of Netrin-1, UNC5 proteins mediate the chemorepulsive effect of Netrin-1 [171]. In addition, UNC5 proteins have been proposed to function as pro-apoptotic "dependence receptors" that trigger apoptosis in the absence of their ligand [197].

UNC5A has also been shown to promote apoptosis independently of Netrin-1, suggesting the possible existence of other functional ligands for this receptor [198, 199]. Loss of UNC5 gene expression has been associated with various cancers and tumor aggressiveness, supporting a role for UNC5 proteins as tumor suppressors (Table 1.4) [200]. Expression studies in mice have shown that UNC5 receptors are expressed in early eye development, mammary bud formation, vascularization and limb development [201], implying that they possess functions outside of the nervous system.

Table 1.4: Alterations of Netrin-1 and UNC5 receptors in cancer.

Gene	Locus	Alterations in cancer	References
UNC5A	5q35.3	LOH in colorectal cancer. Downregulated expression in ovarian, breast, uterine, colorectal, stomach, lung and kidney cancers.	Thiebault, K. et al. 2003
UNC5B	10q21–22	LOH in colorectal cancer. Downregulated expression in ovarian, breast, uterine, colorectal, stomach, lung and kidney cancers.	Thiebault, K. et al. 2003
UNC5C	4q21–23	LOH and homozygous deletions in colorectal cancer. Downregulated expression in ovarian, breast, uterine, colorectal, stomach, lung and kidney cancers.	Thiebault, K. et al. 2003
UNC5D	8p12	LOH and promoter hypermethylation in renal cell cancer. Downregulated expression in x-axis, breast, colon , glioblastoma, renal, lung, pancreatic, rectal cancers.	Lu D, et al. 2013 Dancey JE , et al. 2012
Netrin-1	17p12–13	Downregulated expression in brain tumours and neuroblastomas, and in prostate cancer. Missense mutations in a neuroblastoma.	Meyerhardt, J. A. et al.1999, Latil, A. et al.2003

1.6.1 Structure of human UNC5 receptors

The human genes UNC5A, UNC5B, UNC5C and UNC5D encode receptors for Netrin-1 that are 963-, 945- , 931- and 953-amino acid single-pass type I transmembrane proteins, respectively. All four proteins contains two immunoglobulin repeats followed by two thrombospondin type I repeats in the extracellular domain of the protein [202]. In the intracellular region, UNC5 contains a PEST Zona Occludens-1 homology domain (ZU-5), a DCC binding domain, and a death domain (DD). UNC5-mediated apoptosis occurs via the ZU5 or death domain (Figure 1.9) [199, 203].

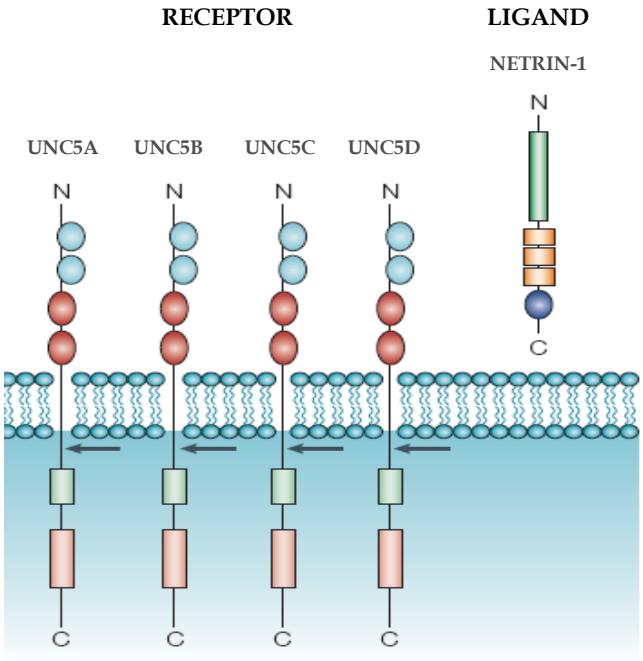

Figure 1.9: Structure of human UNC5: The human UNC5 genes encode type I transmembrane proteins. All four proteins have two immunoglobulin-like domains (indicated by light blue circles) and two thrombospondin-like repeats (indicated by red circles) in the extracellular region, and a zonula occuldens-1 domain (shown as light green rectangles) and a death domain (pink rectangles) in the intracellular region. Adapted from Arakawa, H., et al. Nat Rev Cancer, 2004.

1.6.2 *Unc5* expression during murine development

The expression of *Unc5* genes during mouse development has been examined by RNA in situ hybridization. Whereas *Unc5a* expression is restricted to the ventral spinal cord, *Unc5b* is the most widely expressed and is observed in the developing eye, inner ear, vasculature and limb buds. *Unc5c* expression is strongest in migrating neural crest cells, whereas *Unc5d* expression occurs within the developing limb and mammary gland (Figure 1.10 and Figure 1.11) [201]. The multiple-tissue expression pattern of the *Unc5h* genes during murine development provides clues about the function of UNC5 in

the morphogenesis of various organs.

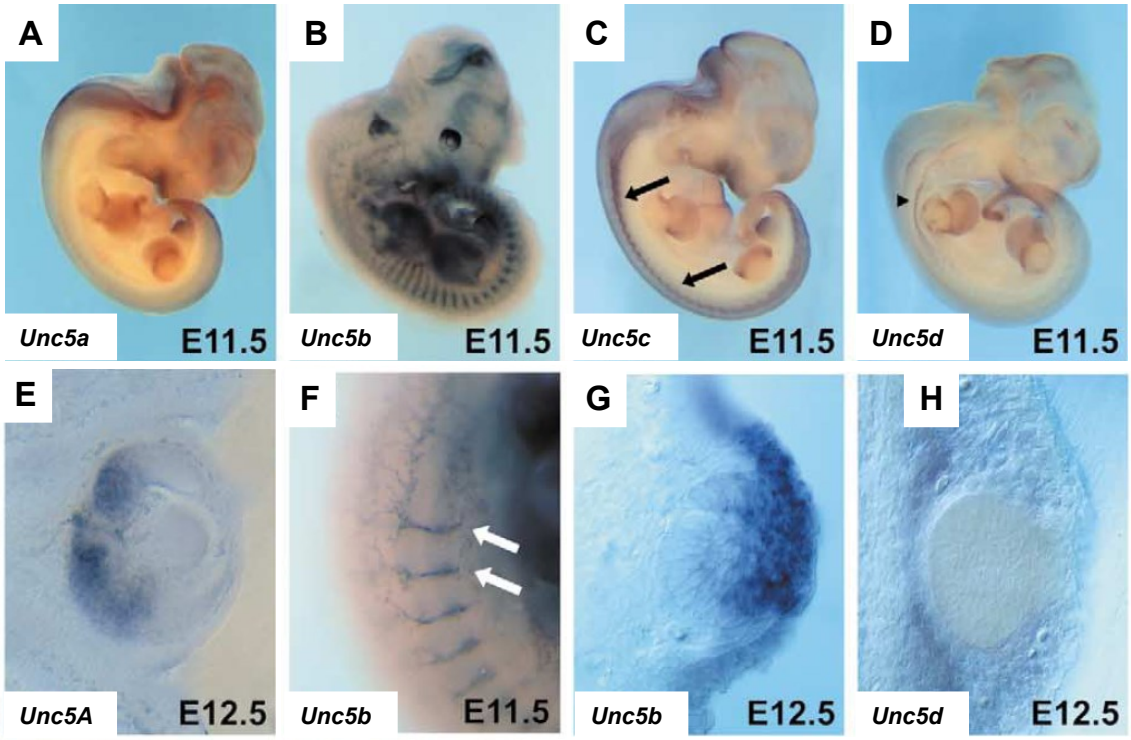

Figure 1.10: Expression patterns of the *Unc5* gene. (A) *Unc5a* expression is restricted to the ventral spinal cord. (B) *Unc5b* expression occurs in the developing eye, inner ear, vasculature and limb buds. (C) *Unc5c* expression is strongest in migrating neural crest cells. (D) *Unc5d* expression is restricted to a narrow region at the base of the forelimb and to the limb bud. (E) *Unc5a* was detected in the central area of the developing eye. (F) *Unc5b* mRNA is noticeable at sites of vascularization. During mammary development (G, H) *Unc5b* and *Unc5d* are expressed at various stages of bud formation. Adapted from Engelkamp, D., Mech Dev, 2002.

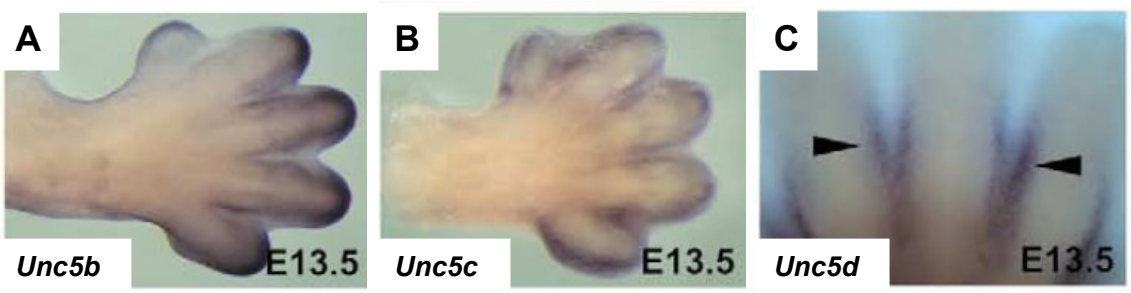

Figure 1.11: Expression patterns of the *Unc5* gene during limb development. (A) *Unc5b* is expressed in the distal limb bud and the interdigital mesenchyme. (B) *Unc5c* is expressed in two areas of the proximal limb bud, the interdigital mesenchyme and the mesenchyme surrounding the fingertips. (C) *Unc5d* was detected at the anterior and posterior sides of the proximal limbs and in proximal peridigital zones. Adapted from Engelkamp, D., Mech Dev, 2002.

1.6.3 UNC5 receptors have a pro-apoptotic function

UNC5 proteins are dependence receptors that produce a survival signal in the presence of Netrin-1, whereas they induce apoptosis when uncoupled from ligand. UNC5 proteins all contain a putative caspase-3 cleavage sequence, DXXD (where X is any amino acid), in the intracellular region [204, 205]. This suggests that caspase cleavage plays an important role in the regulation of the pro-apoptotic function of UNC5 receptors. The death domain is crucial for UNC5-induced apoptosis [203]. The existing evidence shows that UNC5A to D are all cleaved by caspase-3 in vitro and that the cleaved intracellular part of the receptor, including the death domain, may translocate into the nucleus (Figure 1.12) [206, 207].

Although the mechanism of UNC5's pro-apoptotic function is still largely unknown, each UNC5 family member appears to induce cell death by a different apoptotic signaling pathway. The neurotrophin receptor-interacting melanoma-associated antigen homologue (NRAGE) was identified as a UNC5A-interacting protein and shown to

mediate UNC5A-induced apoptosis through degradation of the caspase inhibitor XIAP or activation of the JNK signaling pathway [199]. Death-associated protein kinase (DAPK) might mediate UNC5B-induced apoptosis by interacting with the death domain of the receptor and activating caspase-9 and caspase-3 [208]. UNC5D was recently shown to induce apoptosis through translocation of the caspase-cleaved intracellular fragment into the nucleus and interaction of this fragment with the E2F1 transcription factor [209].

Blocking of the pro-apoptotic effects of UNC5 occurs through dimerization of the receptors when bound to Netrin-1. This dimerization results in structural changes in the intracellular part of the receptor, including the crucial death domain, that prevent caspase cleavage of the receptor [210].

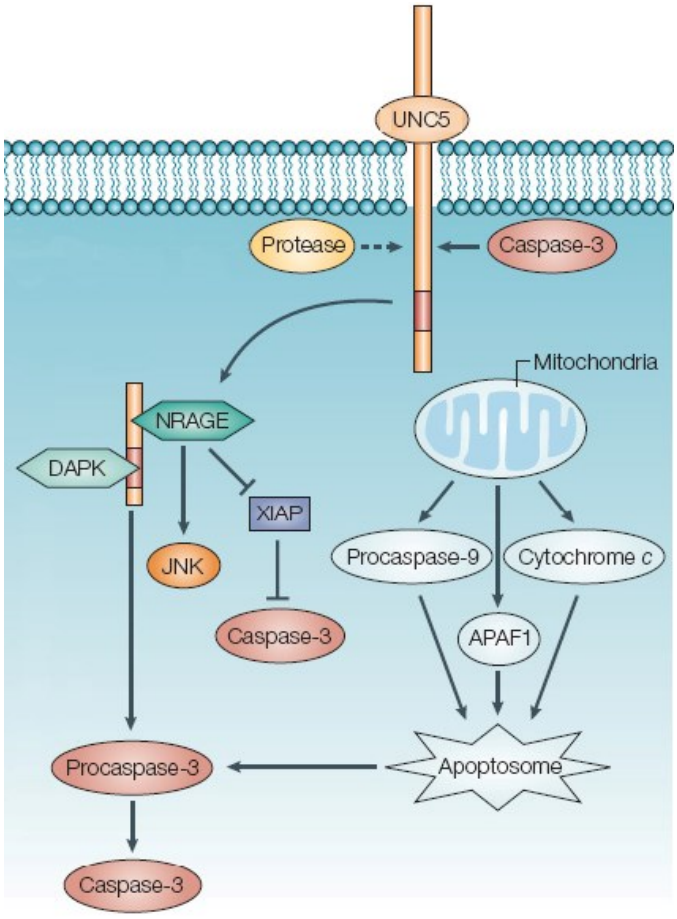

Figure 1.12: Pro-apoptotic function of UNC5. Cleavage of UNC5 at Asp412 by caspase-3 releases the death domain from the C-terminal region. The released peptide

might induce apoptosis through interaction with DAPK and activation of caspase-9 and caspase-3, or it might interact with NRAGE, inducing apoptosis through degradation of the caspase inhibitor XIAP or activation of the JNK signaling pathway [211]. Adapted from Arakawa, H., et al. Nat Rev Cancer, 2004.

1.6.4 UNC5 and p53-dependent apoptosis

The p53 gene has been found to be mutated in more than half of all human cancers and is considered the most important tumor suppressor gene known. The p53 protein plays a role in apoptosis, genomic stability, and inhibition of angiogenesis through the transcriptional activation of target genes, and p53-dependent apoptosis plays a key role in tumor suppression. UNC5B, also termed p53RDL1, was initially reported to be a direct target gene of p53 and to mediate p53-dependent apoptosis in non-neuronal cells [212]. UNC5D (and, recently, UNC5A) were also identified as p53 target genes and shown to be involved in the p53-dependent apoptotic response to DNA damage [213]. UNC5 proteins seem induce apoptosis independent of the mitochondrial and death-receptor pathways, thereby implying the existence of a third pathway for p53-dependent apoptosis. Evidence shows that UNC5-induced apoptosis may depend not only on the cell type but also the p53 status of the cell. For example, in the wild-type p53 cell lines LS174T (colon cancer) and SH-SY5Y (neuroblastoma), all three UNC5s were shown to strongly induce apoptosis. In SKNAS (neuroblastoma) and U373MG (glioblastoma) cells, which contain mutant p53, UNC5B was able to strongly induce apoptosis, whereas UNC5A and UNC5D were not. Thus, the cell's p53 status probably affects UNC5A- and UNC5D-induced apoptosis. Interestingly, Netrin-1 is also a target of p53 [211, 214]. Thus, p53 seems to determine cell fate through the transcriptional regulation of Netrin-1 and UNC5. To remove cells suffering from DNA damage, p53 activates the transcription of UNC5 and induces apoptosis, whereas to

allow cells to survive, p53 activates the transcription of Netrin-1 and UNC5, sending a survival signal. Netrin-1 might block p53 posttranslationally, resulting in inhibition of p53-dependent apoptosis.

1.6.5 UNC5A protein

UNC5A protein, a 963-amino acid single-pass type I transmembrane protein, belongs to the UNC5 protein family. Like other UNC5 family members, UNC5A contains two immunoglobulin repeats followed by two thrombospondin type-I repeats in its extracellular domain [202]. The intracellular region of UNC5A contains a PEST zona occludens-1 homology domain (ZU-5), a DCC-binding domain, and a death domain (DD) (Figure 1.13).

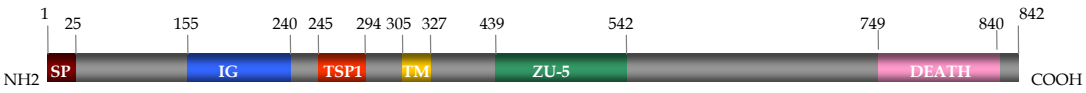

Figure 1.13: The structure of UNC5A protein. The numbers correspond to the amino acid residue beginning at the amino terminus. SP: signal peptide; IG: immunoglobulin-like domains; TSP1: thrombospondin-like repeats; TM: transmembrane region; ZU-5: zonula occuldens-1 domain; DEATH: death domain in the intracellular region.

Loss or downregulation of the UNC5A gene has been found in a variety of cancers including colorectal, breast, ovarian, uterine, stomach, lung, and kidney cancers, supporting the idea that UNC5 proteins function as tumor suppressors [200]. As dependence receptor, when uncoupled from Netrin-1 UNC5A is cleaved at Asp412 by caspase-3, releasing its death domain from the C-terminal region. The released peptide might interact with NRAGE (I34), inducing apoptosis through degradation of the

caspase inhibitor X-chromosome-linked inhibitor of apoptosis protein (XIAP) or activation of the pro-apoptotic c-JUN N-terminal kinase (JNK) signaling pathway [199]. UNC5A has also been shown to promote apoptosis independently of Netrin-1, suggesting the possible existence of other functional ligands for this protein. [197].

So far, UNC5A has been shown to function as a regulator of axon guidance and also to play a role during apoptosis. However, the association of UNC5A with developmental disease or tumorigenesis and the molecular mechanisms through which this might occur remain to be elucidated.

CHAPTER 2: PROJECT HYPOTHESIS AND OBJECTIVES

Current evidence shows that the FA pathway is involved in genome surveillance through a mechanism involving the ubiquitylation of FANCD2 and FANCI and associated with resistance to mitomycin C (MMC) [88, 215]. However, additional data suggest that FA proteins have functions in addition to DNA damage signaling that could explain some of the defects in hematopoiesis and congenital malformations that are associated with FA. For instance, FANCC, a protein that is predominantly located in the cytoplasm, has multifunctional roles, especially as an anti-apoptotic regulator [128, 145, 150, 154, 216].

To better understand the biological functions of FA proteins, a yeast two-hybrid screen was conducted using FANCC proteolytic fragments. Of the clones obtained, one was identified as UNC5A. UNC5A belongs to the UNC5 family of proteins (human UNC5A-D and rodent UNCH1-4), which are receptors for the axon guidance molecule, Netrin-1[186, 217]. They are known as 'dependence receptors' because they positively promote apoptosis in the absence of their ligand, Netrin-1, but negatively promote it when bound to ligand [203, 218-220]. The main objective of this project is the identification of the biological significance of the FANCC/UNC5A interaction and determination of how disruption of this interaction could lead to the clinical phenotypes observed in patients with Fanconi anemia. My working hypothesis is that FANCC, as an anti-apoptotic regulator, has a role in developmental processes that depends on its interaction with UNC5A. To address my hypothesis, I propose the following specific objectives:

2.1 Determine the role of UNC5A-FANCC interaction in apoptosis

A common clinical manifestation in patients with FA is the presence of

life-threatening hematologic abnormalities, including aplastic anemia (AA) that ultimately progresses to full-blown pancytopenia [7, 19-21]. FA patients also have a predisposition to multiple neoplasias, including leukemias and solid tumors. The existence of these phenotypes indicates that FA proteins have other functions in addition to their role in the DNA damage signaling pathway, one possibility being a role in the regulation of apoptosis.

FANCC has been shown to bind the protein chaperones GRP94 and HSP70 [110, 154], the transcription factors FAZF and STAT1 [150, 159], the protein scaffold spectrin II and cdc2 [221-223], which is involved in cell cycle regulation. The interaction between FANCC and these proteins implies that FANCC has cellular roles other than DNA repair. During apoptosis, FANCC undergoes proteolytic modification by a caspase, leading to the production of a predominant 47-kDa ubiquitinated protein fragment [118]. Furthermore, both FANCC and FANCG have been shown to bind non-nuclear proteins involved in oxygen radical metabolism, such as cytochrome P450 reductase, glutathione S-transferase P1, cytochrome P450 2E1 and peroxiredoxin 3 [128-130, 132]. In addition, FANCA, FANCC and FANCG functionally interact with PKR, which plays a critical role in cell growth and apoptosis [224]. All these data further suggest that at least some FA proteins have functions other than those associated with DNA repair.

The UNC5 proteins contain death domains in their intracellular regions that are crucial for UNC5-induced apoptosis [203]. UNC5A-D are all cleaved by caspase-3, and this modification results in the release of the death domain from the C-terminal region of the protein. The released peptide interacts either with the death-associated protein kinase (DAPK), thereby inducing apoptosis through the activation of caspase-9 and caspase-3, or with the neurotrophin receptor-interacting melanoma-associated antigen (MAGE) homologue NRAGE, thereby inducing apoptosis through degradation of the

caspase inhibitor X-chromosome-linked inhibitor of apoptosis protein (XIAP) or through activation of the c-JUN N-terminal kinase (JNK) signaling pathway [199, 203, 205] (Figure 2). UNC5A, UNC5B and UNC5D have all been shown to be direct transcriptional targets of p53 and to mediate apoptosis in a p53-dependent manner [212, 213].

FANCC has been found to directly interact with UNC5A in the Y2H system. Determination of how FANCC, a survival protein, impacts the UNC5A-induced apoptosis pathway will contribute to our understanding of the molecular events underlying the clinical manifestations of FA.

2.2 Determine the functional role of the UNC5A-FANCC interaction in developmental processes

Fanconi anemia is a developmental deficiency disease. In adult Fanconi mice, reduced proliferation of neural progenitor cells related to apoptosis and accentuated neural stem cell exhaustion with aging were observed [225, 226]. In addition, embryonic and adult Fanconi neural stem cells showed a reduced capacity to self-renew in vitro. When bound to Netrin-1, UNC5A mediates a repulsive signal that affects neural development and differentiation [198, 227-229]. Furthermore, members of the UNC5 protein family are involved in the morphogenesis of a variety of tissues including lung, mammary gland and vascular networks [195, 201, 230, 231]. Studies of the expression of *Unc5* genes during mouse development showed that *Unc5A* expression is restricted to the central nervous system (CNS) [201] whereas *FancC* was detected in the mesenchyme of the spinal cord and in the actively dividing ependymal cell layer of the developing brain [121]. The overlapping expression patterns of *FancC* and *Unc5A* in CNS imply a possible role of UNC5A-FANCC interaction in development and neurogenesis.

2.3 Characterize the interplay between FA and UNC5 signaling pathways

Each of the FA core complex proteins, including FANCC, is required for the integrity and nuclear localization of the FA core complex. As a direct partner of FANCC, UNC5A might interact with other FA proteins, function in core complex integrity and/or play a role in the FA pathway. UNC5A has been found to be lost or downregulated in multiple cancers, including colorectal, breast, ovarian, uterine, stomach, lung, and kidney cancers, suggesting a role of UNC5A protein as a tumor suppressor [200]. FANCC has been shown to interact with UNC5A and might function as a regulator of the molecular events associated with the expression of UNC5A. Under FA disease conditions in which the FANCC-UNC5A interaction is disrupted, both the FA signaling pathway and the UNC5 signaling pathway are influenced.

Although the FA pathway has been clearly implicated in the signaling events associated with DNA crosslinking damage, the functions of the FA proteins are still unknown, especially with respect to how these proteins might be involved in some of the disease phenotypes, stem cell defects and congenital malformations. As dependence receptors, the UNC5 family of proteins has been implicated in developmental processes through its role in apoptosis. The direct interaction between FANCC and UNC5A suggests that crosstalk occurs between the UNC5 and FA pathways. My project will help determine the functional relationship between these two pathways and contribute to our understanding of the functions of FA proteins and the FA pathway in development.

CHAPTER 3: RESULTS-ARTICLES I

The Fanconi anemia group C protein interacts with uncoordinated 5A and delays apoptosis

(Published: March 27, 2014. PLoS ONE 9(3): e92811.)

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In this paper, I (Fengfei Huang) performed the most experiments showed in Figure 3.1, 3.2, 3.4, 3.5 and 3,6. Caroline Huard did the Y2H screen, results showed in Table 3.1 and 3.2. Manel did parts of Figure 3.5 and 3.6. Audrey and Chantal Godin contributed to Fig 3.3. I wrote the paper draft and my supervisor (Dr. Madeleine Carreau) corrected before submission.

3.1 Abstract

The Fanconi anemia group C protein (FANCC) is one of the several proteins that comprise the Fanconi anemia (FA) network involved in genomic surveillance. FANCC is mainly cytoplasmic and has many functions, including apoptosis suppression through caspase-mediated proteolytic processing. Here, we examined the role of FANCC proteolytic fragments by identifying their binding partners. We performed a yeast two-hybrid screen with caspase-mediated FANCC cleavage products and identified the dependence receptor uncoordinated-5A (UNC5A) protein. Here, we show that FANCC physically interacts with UNC5A, a pro-apoptotic dependence receptor. FANCC interaction occurs through the UNC5A intracellular domain, specifically via its death domain. FANCC modulates cell sensitivity to UNC5A-mediated apoptosis; we observed reduced UNC5A-mediated apoptosis in the presence of FANCC and increased apoptosis in FANCC-depleted cells. Our results show that FANCC interferes with UNC5A's functions in apoptosis and suggest that FANCC may participate in developmental processes through association with the dependence receptor UNC5A.

3.2 Introduction

The Fanconi anemia group C protein (FANCC) is a multifunctional protein, with roles in several cellular processes, such as DNA damage signaling, redox regulation, transcriptional regulation, and apoptosis [1]. Mutations in the *FANCC* gene lead to Fanconi anemia, a genetic disease characterized by a progressive depletion of bone marrow cells [2,3]. This disease is also associated with various congenital malformations

and an increased risk of malignancies [4]. There are 16 FA disease-associated genes that form an entity known as the FA pathway that enacts a global response to DNA crosslink damage [5-7]. FANCC is the cytoplasmic component of the FA pathway, and in association with the Fanconi anemia group E (FANCE) protein, translocates to the nucleus in response to crosslink damage [8-10]. Nuclear FANCC associates with other components of the FA pathway to compose the FA core complex. Besides this association with FA core complex proteins, FANCC binds several proteins involved in various cellular functions, such as oxygen radical metabolism, signal transduction, transcription, and apoptosis [1,11]. FANCC has mostly been studied in the context of cell survival and death signaling. For instance, FANCC-deficient cells show increased apoptosis in response to inhibitory cytokines, serum deprivation, apoptosis inducers, DNA crosslink damage, and reactive oxygen species [12-14]. FANCC over-expression attenuated apoptosis and induced a survival response in non-FA cells, thus, FANCC is considered a survival or anti-apoptotic protein [12,14-20]. We have previously shown that in response to apoptosis, FANCC undergoes caspase-mediated proteolytic processing, leading to the generation of cleaved protein fragments [15]. Cleaved FANCC is not able to suppress apoptosis, but a non-cleavable form of FANCC further delays its onset [15]. Currently, little is known about the molecular events leading to FANCC cleavage and its impact on downstream cellular signaling. To further characterize the cellular functions of FANCC, we performed yeast two-hybrid screens using FANCC cleavage products to identify protein interactors. Among the candidates obtained, one candidate coded for the dependence receptor uncoordinated-5 A (UNC5A).

UNC5A is a member of the Netrin-1 transmembrane receptor family that is comprised of four homologs, namely UNC5A, UNC5B, UNC5C, and UNC5D, also called UNC5H1, UNC5H2, UNC5H3, and UNC5H4. The UNC5 receptors are single-pass type I transmembrane proteins that contain two immunoglobulin repeats followed by two thrombospondin type-I repeats in the extracellular domain [21]. The intracellular region of UNC5A contains a PEST zona occludens-1 homology domain (ZU-5), a deleted in colorectal cancer (DCC)-binding domain, and a death domain (DD). UNC5 proteins have been proposed to function as proapoptotic "dependence receptors" that trigger apoptosis in the absence of their ligand [22]. UNC5-mediated apoptosis occurs via the ZU5 domains or DDs [23,24]. Expression studies in mice have shown that UNC5 receptors are expressed in early eye development, mammary bud formation, vascularization, and limb development [25]. In addition, loss of UNC5 gene expression is associated with various cancers and tumor aggressiveness, supporting the hypothesis that UNC5 proteins act as tumor suppressors [26].

Here, we show a direct interaction between FANCC and UNC5A cytoplasmic death domain. We also show that FANCC delays UNC5A-mediated apoptosis.

3.3 Materials and Methods

Plasmids and DNA constructs.

The N-terminus of FANCC, which spans from nucleotides 256 to 1175 and encompasses amino acids from the start codon to the cleavage site [15], was cloned into the pGBKT7 and pGADT7 yeast vectors (Clontech Laboratories Inc., Mountain View,

CA) by fusion to the Gal4-DNA binding or DNA-activating domain, and into the pEGFP plasmids (pGBKFANCC$^{1-306}$, pGADFANCC$^{1-306}$, pEGFPFANCC$^{1-306}$). Similarly, the C-terminus of FANCC, spanning from nucleotides 1176 to 1929 and corresponding to the cleaved FANCC C-terminus fragment amino acids 307 to 558, was cloned into the pGBKT7 and pGADT7 yeast vectors (pGBKFANCC$^{307-558}$ and pGADFANCC$^{307-558}$) and the pEGFP plasmid (pEGFPFANCC$^{307-558}$), as previously described [15]. The pGADT7-FANCC$^{307-558-L554P}$ and pGBKT7-FANCC$^{307-558-L554P}$ constructs were obtained by site-directed mutagenesis using the FANCC$^{307-558}$ coding plasmids and the Quikchange II site-directed mutagenesis kit according to the manufacturer's protocol (Agilent Technologies, Mississauga, ON). Other FA gene constructs have been described previously [27]. The Myc-tagged rat UNC5A plasmid (pSecrUnc5A) was generously provided by Dr. Tessier-Lavigne. Upon sequence verification of this plasmid, we noticed that part of the 5' end (118 bp) and 1000 bp of the 3' end of the full length Unc5A cDNA was missing. We therefore cloned the full-length human UNC5A and the intracellular domain of the human UNC5A spanning from nucleotides 985 to 2529 of the open reading frame (corresponding to amino acids 329 to 842) by RT-PCR into pCMVzeo vectors in frame with an HA-tag (pCMVzeoUNC5A^{ICD}). Subsequently, UNC5A and UNC5A$^{329-842}$ (UNC5A^{ICD}) were subcloned into pGBKT7 and pGADT7 yeast vectors and pCDNAHisXpres and pCDH-CMV-MCS lentiviral vectors. UNC5A with a deleted DD (UNC5A$^{\Delta DD}$) was cloned into pGBKT7 and pGADT7 yeast vectors. Lentiviral vectors TRCN0000083368, TRCN0000083369, TRCN0000083370, TRCN0000083371 and TRCN0000083372

coding for shRNA against FANCC; V2LHS-74072, V2LHS-74073, V3LHS-321475, V3LHS-321477, V3LHS-321480 coding for shRNA against FANCE; V2LHS-16512, V2LHS-16513, V2LHS-304038, V2LHS-304039, V2LHS-304040 coding for shRNA against UNC5A were obtained from Thermo Scientific (ThermoFisher Scientific, Mississauga, ON).

Antibodies.

The following antibodies were used: previously described 8F3 anti-FANCC [15], a gift from Dr. M. Hoatlin (OHSU), and Novus Biologicals (Littleton, CO); anti-FANCA (Santa Cruz Biotechnologies, Santa Cruz, CA), anti-UNC5A (Sigma-Aldrich, St. Louis, MO); anti-HA (12CA5, Roche Diagnostics, Indianapolis, IN); anti-cMyc (9E10, Santa Cruz Biotechnologies, Santa Cruz, CA); anti-GFP (clone B2; Santa Cruz Biotechnologies); anti-cleaved-caspase-3 (Cell Signaling Technologies, Danvers, MA), anti-mouse and anti-rabbit (Santa Cruz Biotechnologies); and donkey anti-rabbit Alexa Fluor 488 or 555 and anti-mouse Alexa Fluor 555 or 488 (Invitrogen, Burlington, ON). F-actin was labeled with Alexa Fluor 546 phalloidin (Life Technologies, Burlington, ON).

Yeast two-hybrid screens and analyses.

Yeast two-hybrid screens and analyses were performed using the MATCHMAKER Two-Hybrid System 3 according to the manufacturer's instructions (Clontech). The FANCC N- and C-terminal fragment (pGBKFANCC$^{1-306}$ and pGBKFANCC$^{307-558}$,

respectively) bait constructs were tested for self-activation in the yeast two-hybrid assay prior to use with library screens and were found to be free of autonomous Gal-4 activation. Yeast two-hybrid screens were performed according to the manufacturer's protocol (Clontech) using the MATCHMAKER Two Hybrid System 3, where the bait FANCC constructs were transformed into the AH109 *Saccharomyces cerevisiae* yeast strain containing the nutritional reporter genes *ADE2* and *HIS3*. The transformed AH109 yeasts were mated with the Y187 *S. cerevisiae* strain, which was pre-transformed with cDNA libraries obtained from either HeLa cells or human fetal brain (Clontech). Yeast diploids were selected by plating on dropout medium lacking tryptophan and leucine (-TL), and by detecting protein-protein interactions on dropout medium lacking tryptophan, leucine, histidine, and adenine (-TLHA). Three different screens were performed using each pGBKT7-FANCC$^{1\text{-}306}$ and pGBKT7-FANCC$^{307\text{-}558}$ construct as the bait.

For yeast two-hybrid analyses using FA proteins, all FA genes were subcloned into yeast two-hybrid vectors, as previously described [27]. Constructs were co-transformed into the AH109 *S. cerevisiae* strain (Clontech) and selected for growth and reporter gene activation. Positive controls included pGBKT7-p53 with the pGADT7-T antigen and pGBKT7-FANCF with pGADT7-FANCG. Negative controls were empty pGBKT7 or pGADT7 vectors in combination with the corresponding FA gene-coding plasmid. Each construct was sequenced and tested for autonomous Gal-4 activation, and FANCG and FANCL showed self-activation when cloned into pGBKT7 [27,28]. Each experiment was performed at least three times in triplicate and with each gene cloned into either the

pGBKT7 or pGADT7 vectors.

Cells, cultures, and transfection.

HEK293T and HeLa cells were grown at 37°C, 5% CO2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). SH-SY5Y cells were grown at 37°C, 5% CO2 in a 1:1 mixture of DMEM and Ham's F12 nutrient mixture (HyClone, ThermoFisher Scientific) and 10% FCS. Cells were transfected using the calcium-phosphate method or Lipofectamine 2000 (Invitrogen). For cellular depletion, SH-SY5Y cells were transduced with lentiviral particles coding for a mixture of 5 different shRNA against FANCC or UNC5A or scrambled sequences. Lentiviral particles were produced using the four-plasmid expression system containing pRSV-Rev, pMDLg/pRRE, pMD2.G and the different expression vectors as previously described [11]. Following transduction, cells were selected and maintained in media containing puromycin (2.0 ug/ml, Life technologies, Burlington, ON). For induction of neurite outgrowth, SH-SY5Y cells were plated in collagen-coated 6-well plates at a density of 2 X 10^4 cells/cm^2 and were deprived of serum (0.5% FCS) for 24 to 48 hours and treated with retinoic acid (10 μM, Sigma-Aldrich) or DMSO for up to 6 days or treated with staurosporin (25 nM, Sigma-Aldrich) for 72 hours. Cells were treated with recombinant human Netrin-1 (500 ng/ml; R&D systems, Minneapolis, MN) for 4 hours prior to immunofluorescence staining. For neurite length estimation, cells were visualized using a Nikon E300 inverted microscope (Nikon Canada, Mississauga, ON) at 40X magnification. At least 200 cells from microscopic fields selected at random were

counted in each sample.

Immunoblot analysis and immunoprecipitations.

For immunoblot analysis, total cell lysates were prepared in sodium dodecyl sulfate (SDS)-loading buffer (50 mM Tris-HCl, 2% 2-mercaptoethanol, 2% SDS). Samples were sonicated and/or boiled and subjected to electrophoresis on 10% or 12% SDS-polyacrylamide gels. Proteins were electrotransferred onto a PVDF membrane (Amersham) and probed with antibodies, as indicated in each figure. For immunoprecipitation (IP), 5×10^6 to 1×10^7 cells were harvested, washed in phosphate-buffered saline (PBS), and resuspended in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, and complete proteases inhibitors [Roche Diagnostics]). Lysates were cleared by centrifugation and mixed with 1 to 2 μg of the precipitating antibody, as indicated in the figure. Antibody-antigen complexes were pulled down with protein A-agarose beads (Calbiochem, San Diego, CA) for over-expressed proteins or Dynabeads (Invitrogen) for endogenous protein complexes. Immunoprecipitates were resolved by SDS-polyacrylamide gel electrophoresis on 10% or 12% polyacrylamide gels and subjected to Western blotting with specific antibodies, as indicated in each figure. Control IPs were performed using either mouse or rabbit IgGs, as indicated in the figures.

Immunofluorescence microscopy.

For localization of FANCC and UNC5A, immunofluorescence microscopy was

performed as follows. SH-SY5Y cells were grown in the appropriate culture condition on poly-L-lysine coated coverslips (12-mm diameter) prior to treatment. Cells were fixed with either 4% paraformaldehyde in PBS for 20 minutes at room temperature or methanol/acetone (3:7) for 20 minutes at -20˚C, followed by permeabilization for 15 minutes at room temperature with 0.3% Triton X-100 in PBS or 1 hour with 0.1% saponin with 2% BSA in PBS. Fixed cells were incubated with specific primary antibodies as indicated in the figures, followed by secondary antibodies (goat anti-mouse Alexa Fluor 488, goat anti-rabbit Alexa Fluor 546, or donkey anti-rabbit Alexa Fluor 555) at the appropriate dilution in PBS with 10% horse serum or with 0.1% saponin with 2% BSA. Following labeling with primary and secondary antibodies, cells were washed three times with PBS. Images were acquired using a Nikon E800 fluorescent microscope equipped with a C1 confocal system (Nikon Canada, Mississauga, ON).

Apoptosis assays.

Apoptosis was induced in SH-SY5Y cells transfected with the appropriate DNA constructs by 4-hour staurosporin treatment (1 μM; Roche Diagnostics). Following apoptosis induction, caspase-3 activation was assessed with a Caspase-3, Active Form, mAb Apoptosis Kit according to manufacturer's instructions (BD Biosciences, Mississauga, ON). Caspase-3-positive cells were analyzed by confocal microscopy and manually scored for both GFP-positive (transfected cells) and caspase-3-positive cells, or analyzed by flow cytometry (BD SORP LSR II; BD Biosciences) by gating for GFP-

and caspase-3-positive cells.

Statistical analyses.

Data were expressed as means ± standard errors of the means (SEMs). Statistical analyses were performed using the GraphPad Prism software (version 5.0b; GraphPad Software Inc., San Diego, CA), and the paired and unpaired two-tailed Student's t-tests were used to compare the means. P values less than 0.05 were considered significant.

3.4 Results

Identifying novel partners of FANCC.

To gain further insight into FANCC's role in apoptosis we performed yeast-2-hybrid screens with FANCC's caspase-mediated proteolytic fragments. We used both N-terminal ($FANCC^{1-306}$) and C-terminal ($FANCC^{307-558}$) regions of FANCC corresponding to cleavage products as baits for yeast two-hybrid analysis. Two separate and independent screens were performed using a fetal brain cDNA library as prey. Most protein candidates were obtained with screens using $FANCC^{1-306}$ as bait (Table 1). Among the positive yeast colonies that were obtained with $FANCC^{1-306}$, two independent and strong positive candidates encoded the C-terminal portion of the UNC5A protein (amino acids 729 to 842). Of the candidates obtained, one had been previously identified by another group, notably, the FANCG-interacting protein peroxiredoxin-3 (clone 5.6) [29], whereas the C-terminal-binding protein 1 (clone 11.1) has been previously published by us [11]. All clones were retested in yeast two-hybrid

assays against FANCC$^{1-306}$ or the empty bait vector (Table 2). Eight of the eleven clones that were retested in yeasts assays, including UNC5A, showed positive interaction with FANCC$^{1-306}$, whereas three candidates did not show interaction with FANCC$^{1-306}$ when plated on selective media (-TLHA).

FANCC interacts with UNC5A.

We selected the UNC5A candidate and subcloned it into pGBKT7 and pGADT7 yeast vectors. These we subsequently tested in yeast two-hybrid assays against FANCC$^{1-306}$, FANCC$^{307-558}$ and the full-length FANCC protein. Results showed that the UNC5A clone corresponding to the C-terminal region directly interacts with FANCC$^{1-306}$, as well as with full-length FANCC but not with FANCC$^{307-558}$ (Figure 1). Sequencing analysis revealed that the UNC5A clone contained intronic sequences (129 bp upstream the exon 15 splicing site) corresponding to parts of intron 14 of the *UNC5A* gene (Figure 1, shown in blue). Therefore, we generated yeast vectors containing cDNA corresponding to the intracellular domain of the UNC5A protein (UNC5A^{ICD}), to the death domain deletion mutant (UNC5A$^{\Delta DD}$) and to the C-terminus part containing the death domain (UNC5A^{DD}).

As shown by yeast two-hybrid analysis, the intracellular domain of UNC5A (UNC5A^{ICD}) showed positive interaction with FANCC$^{1-306}$ but not full-length FANCC or FANCC C-terminus domain (FANCC$^{307-558}$). However, results show that the UNC5A C-terminus containing the death domain interacts directly with full-length FANCC and both FANCC fragments. In addition, FANCC$^{1-306}$ but not full length FANCC or

FANCC$^{307-558}$ showed positive interaction with UNC5A lacking the death domain (UNC5A$^{\Delta DD}$). All UNC5A and FANCC constructs were found devoid of self-activation as shown by negative growth with empty vectors on stringent nutritional selection (Figure 1). These results suggest that FANCC$^{1-306}$ binds UNC5A via more than one region, whereas FANCC$^{307-558}$ and full-length FANCC interacts with UNC5A death domain. Next, to determine whether a FA-causing mutation of FANCC impacted its ability to interact with UNC5A, we generated a FANCC C-terminus construct harboring the L554P mutation (FANCC$^{307-558-L554P}$) found in patients with FA. Results showed that the interaction between the UNC5A C-terminus and FANCC$^{307-558-L554P}$ still occurred in yeasts. These results imply that a mutated FANCC protein may conserve interaction with UNC5A.

FANCC immunoprecipitates with UNC5A.

To determine whether FANCC interaction with UNC5A occurs in cells, we first performed immunoprecipitation with cell extracts overexpressing UNC5A and FANCC. To do so, we obtained the rat UNC5A cDNA coding vector pSecUNC5A (generously provided by Dr Tessier-Lavigne) and performed coimmunoprecipitation experiments (Figure 2A). Although FANCC coimmunoprecipitated with the rat UNC5A protein, sequencing analysis of this plasmid revealed a partial open-reading frame where 118 bp were missing from the 5' end and 1000 bp missing from the 3' end, which encodes the death domain. However, these results supports data obtained in yeasts assays showing that UNC5A interaction with FANCC occurs through different UNC5A protein domains.

Next, we amplified the human cDNA of the intracellular domain (ICD) of UNC5A and cloned it into mammalian expression vectors. We then performed co-immunoprecipitation studies in cells expressing the HA-tagged UNC5A^{ICD} and FANCC. Immunoprecipitation was performed with either anti-HA or anti-FANCC antibodies. Immunoblot analyses revealed that full-length FANCC co-immunoprecipitates with UNC5A^{ICD} (Figure 2B). Immunoprecipitation performed with HA-tagged UNC5A^{ICD} and endogenous FANCC confirms results obtained with overexpressed proteins (Figure 2C).

Next, we performed co-immunoprecipitation studies in cells expressing EGFP-tagged FANCC$^{1-306}$ or FANCC$^{307-558}$ with UNC5A^{ICD} by using anti-GFP and anti-HA (HA-UNC5A^{ICD}) antibodies. Western blot analyses showed that the immunoprecipitates contained UNC5A^{ICD} and the FANCC N-terminus (Figure 2D, lower panel) or the C-terminus regions (Figure 2E, lower panel). Together, these results and data obtained in yeasts suggest that FANCC protein fragments interact with different UNC5A protein domains.

To determine whether FANCC interaction with UNC5A in cells requires the UNC5A death domain, we performed immunoprecipitation analysis using protein extracts from cells expressing full-length FANCC and a UNC5A death domain deletion mutant (UNC5A$^{\Delta DD}$). Immunoprecipitation were performed with antibodies against the Xpress epitope tag of UNC5A or the HA epitope tag of FANCC. Although results show that FANCC still co-immunoprecipitated with UNC5A$^{\Delta DD}$ only a faint FANCC protein band is detected in the Xpress-mediated IP lane or a faint UNC5A band in the HA-mediated

IP lane (Figure 3A). Because no protein is detected in the control IgG immunoprecipitates, results suggest that a week interaction occurs between FANCC and UNC5A$^{\Delta DD}$. These results also imply that the UNC5A death domain may be required for strong FANCC binding in cells or that a protein present in cells but absent in yeasts mediates FANCC interaction with UNC5A$^{\Delta DD}$. Next, to determine whether full-length FANCC harboring the L554P mutation retained the interaction with UNC5A as in yeasts, we performed immunprecipitation analyses using a HA-tagged full-length FANCC^{L554P} mutant. Results show that a week interaction between FANCC^{L554P} and UNC5A$^{\Delta DD}$ occurred (Figure 3B). Together, these results indicate that FANCC interacts with UNC5A in cells and that the L554P mutation in FANCC does not disrupt this interaction. These results also indicate that the UNC5A death domain is required for strong FANCC binding. As expected, immunofluorescence analysis indicated that endogenous UNC5A is found in the cytoplasm and colocalizes with cytoplasmic FANCC (Figure 4). Collectively, these results indicate that FANCC interacts with UNC5A.

FANCC prevents UNC5A-mediated apoptosis.

UNC5A has been shown to trigger apoptosis following caspase-dependent cleavage and release of its intracellular C-terminal domain (ICD) [30-32]. Because FANCC exhibits anti-apoptotic properties, is cleaved by a caspase and interacts with the intracellular domain of UNC5A [15,33], we sought to determine the mechanistic impact of FANCC on UNC5A-mediated apoptosis. First, we measured apoptosis in cells over-expressing UNC5A with or without FANCC. HeLa cells were transfected with UNC5A^{ICD} and

FANCC, and tested for caspase-3 activation. As expected, UNC5A^{ICD}-over-expressing cells were prone to apoptosis and showed elevated numbers of caspase-3-positive cells whereas cells expressing FANCC with UNC5A^{ICD} exhibited reduced numbers of caspase-3-positive cells (Figure 5A–B). These results suggest that FANCC has a protective role in UNC5A-mediated apoptosis. Next, we evaluated the UNC5A-mediated apoptosis in cells expressing the FANCC protein harboring the L554P mutation found in patients with FA. Results show that the FANCC^{L554P} protein had a dominant negative effect over the endogenous wild-type FANCC and conferred no protective effect in cells expressing the UNC5A^{ICD} protein compared to the wild-type FANCC protein (Figure 5C). These results imply that cells with a defective FANCC protein would become more sensitive to UNC5A-mediated apoptosis. Consequently, we used FANCC-depleted cells to determine the impact of FANCC on UNC5A-mediated apoptosis. SH-SY5Y cells were transduced with lentiviral particles coding for shRNA against FANCC and subsequently, transfected with UNC5A^{ICD}. Depletion of the FANCC protein was confirmed by Western blotting procedures (Figure 6A). Results showed that FANCC depletion as well as UNC5A^{ICD} overexpression increased apoptosis, as evidenced by the elevated number of caspase-3-positive cells (Figure 6B). In addition, expression of UNC5A^{ICD} together with FANCC depletion resulted in a dramatic increase in apoptosis. Together, these results suggest that FANCC modulates the cell sensitivity to UNC5A-mediated apoptosis.

3.5 Discussion

Our results provide the first evidence linking FANCC to UNC5A, a cellular receptor involved in tissue morphogenesis. Currently, very little is known about the role of FA proteins in developmental processes. FA proteins are thought to play a role in tissue homeostasis based on clinical disease phenotypes and developmental expression patterns [34-36]; however, a clear link has been elusive. Our data demonstrate that FANCC, which is also known as an anti-apoptotic protein, directly interacts with the pro-apoptotic dependence receptor UNC5A. Our results show that FANCC over-expression interferes with UNC5A-mediated apoptosis, whereas absence of a functional FANCC protein through depletion or mutation leads to increased UNC5A-mediated apoptosis. Thus, FANCC regulates the cell sensitivity to UNC5A-mediated apoptosis and may therefore act as a sensor of cellular stress and a switch between apoptosis and survival (Figure 7).

Based on expression studies and the role of UNC5A in apoptosis, it is considered to be a physiological regulator of tissue size and shape. For instance, UNC5 homologs are highly expressed in developing limbs [25]. Similarly, FA genes are expressed primarily in cells of mesenchymal origin that give rise to forelimb and hind limb tissues [34-36]. Consequently, defective FA genes result in limb malformations, and patients with FA can show absence or underdevelopment of thumbs and short or hypoplastic radii [3]. In view of our results that FANCC negatively impacts UNC5A's ability to induce apoptosis, we propose that dysregulation of UNC5A's apoptotic signal could lead to developmental defects similar to those observed in FA patients.

It is well known that FANCC suppresses apoptosis; FANCC-depleted cells or

patient-derived cells with *FANCC* mutations show increased apoptosis induced by various cellular stressors, such as DNA damage, inhibitory cytokines, and oxygen radicals, whereas FANCC over-expression delays onset of apoptosis [1]. FANCC is also regulated by caspase-mediated cleavage, which inactivates its ability to suppress apoptosis [15]. The fact that FANCC directly interacts with the UNC5A C-terminal end harboring the DD suggests that interaction of both proteins may interfere with death signal transmission. It has been proposed that UNC5A mediates apoptosis via its C-terminal DD, although conflicting data suggest that the ZU-5 C-terminal domain, which binds the melanoma antigen gene D1 (MAGED1, formerly NRAGE), is required for apoptosis [23,24]. The UNC5A homolog protein UNC5B was shown to directly interact with the death-associated protein kinase (DAPK) via its DD [37,38]. This UNC5B/DAPK interaction was shown to be required for activation of the apoptotic cascade. Although UNC5A did not directly bind DAPK, regulation of DAPK activation via protein phosphatase 2A (PP2A) was shown to be required for UNC5A-mediated apoptosis [37,38]. We identified a direct interaction between FANCC and the C-terminal region of UNC5A, including the ZU-5 and DD, suggesting that FANCC binding with UNC5A may interfere with UNC5A binding to apoptosis-promoting factors, such as MAGED1. Consequently, FANCC may act as a cellular sensor of UNC5A-mediated apoptotic cues and prevent or delay apoptosis depending on the tissue or cellular context.

3.6 Acknowledgment

We are grateful to Dr. Maureen Hoatlin (OHSU) for the 8F3 monoclonal anti-FANCC antibodies and to Dr Tessier-Lavigne for providing the pSecMyc-UNC5A plasmid.

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3.8 Figure Legends

Figure 3.1: FANCC directly interacts with UNC5A in yeasts. Yeast two-hybrid assays were performed with various UNC5A constructs that included the Y2H clone coding for the C-terminal death domain (DEATH) and a part of intron 14 (shown in blue), UNC5A intracellular domain ($UNC5A^{ICD}$), the UNC5A C-terminus ($UNC5A^{DD}$) and the death-domain deletion mutant ($UNC5A^{\Delta DD}$). The yeast strain AH109 was co-transformed with UNC5A constructs along with FANCC constructs as indicated. The plus sign (+) indicate a positive interaction. Negative controls were performed using empty vectors (Empty), and positive controls were performed using pGBKT7/p53 and pGADT7/SV40 T-antigen from Clontech (data not shown). Yeast two-hybrid assays were performed at least three times (independent transformation) with 3 to 4 clones each.

Figure 3.2: FANCC interacts with UNC5A. Immunoprecipitations (IPs) were performed in HEK293T cells transiently transfected with Myc-tagged rUNC5A (A), HA-tagged $UNC5A^{ICD}$ (B-C), with (A-B) or without (C) full-length FANCC. In D and E, HEK293T cells were cotransfected with HA-tagged $UNC5A^{ICD}$ and EGFP-tagged $FANCC^{1-306}$ (D) or EGFP-tagged $FANCC^{307-558)}$ (E). IPs were performed using anti-FANCC, anti-Myc, anti-HA, anti-GFP or control mouse serum (IgG). Western immunoblotting (IB) was performed with the indicated antibodies. WCE: whole cell extract from transfected cells, C: control untransfected cells. * indicates non-specific or IgG bands. Numbers indicate molecular weight.

Figure 3.3: FANCC interacts with UNC5A$^{\Delta DD}$. Immunoprecipitations (IPs) were performed in HEK293T cells transiently transfected with Xpress-tagged death-domain deletion mutant UNC5A (UNC5A$^{\Delta DD}$) and HA-tagged FANCC (A) or in B HA-tagged FANCC harboring the L554P mutation (HA-FANCC^{L554P}). IPs were performed using anti-Xpress, anti-HA or control mouse serum (IgG). Western immunoblotting (IB) was performed with the indicated antibodies. WCE: whole cell extract from transfected cells. * indicates non-specific or IgG bands. Numbers indicate molecular weight

Figure 3.4: FANCC co-localizes with UNC5A in the cytoplasm. Representative immunofluorescence experiment performed in HEK293T cells double-stained with anti-UNC5A (red) and anti-FANCC (green) antibodies. Cells were visualized via confocal fluorescence microscopy using a Nikon E800 microscope equipped with a C1 confocal system at 100X magnification.

Figure 3.5: FANCC interferes with UNC5A-mediated apoptosis. HeLa cells were transiently transfected with EGFP-UNC5A^{ICD} with or without full-length FANCC or empty vectors. At 48 hours after transfection, cells were fixed and labeled with anti-cleaved-caspase-3 antibodies, and visualized by fluorescence microscopy using a Nikon E800 microscope equipped with a C1 confocal system at 60X magnification. (A) Representative immunofluorescence experiment showing EGFP-UNC5A^{ICD} transfected cells (green) and cleaved-caspase-3 positive cells (red). (B) Data are expressed as the mean percent ± standard error of the mean (SEM) of cleaved-caspase-3-positive cells

out of EGFP-positive cells from 3 separate experiments. (C) Data are expressed as ratio of caspase-3-positive cells obtained from EGFP-positive UNC5A^{ICD} cells compared to cells transfected with empty vectors.

Figure 3.6: FANCC-depleted cells are sensitive to UNC5A-mediated apoptosis. SH-SY5Y cells were stably transduced with shRNA against FANCC (sh-C1 and sh-C2) or scrambled shRNA vectors (C). FANCCi and cells transduced with scrambled shRNA (control) were transfected with UNC5A^{ICD} or an empty vector. At 48 hours after transfection, cells were fixed and labeled with anti-cleaved-caspase-3 antibodies, and analyzed by fluorescence microscopy. Data are expressed as the mean cleaved-caspase-3 positive cells ± SEM from three separate experiments performed in duplicate. P values are indicated in the graph.

Figure 3.7: Schematic representation of FANCC interaction with UNC5A and possible role in apoptosis. Based on interactions studies, full length FANCC interacts with UNC5A via different parts of the intracellular domain. Upon apoptosis, FANCC and UNC5A are cleaved by a caspase. Cleaved FANCC fragments interact with UNC5A and interfere with the apoptosis signal (survival). In absence of FANCC or mutations in FANCC, UNC5A triggers apoptosis.

Table 3.1: Candidate clones obtained from Yeast-2-hybrid screens

Clone number	Accession number	Gene	E-value	ID (%)
1.1	NM_001243743.1	FANCC	0.0	62
2.1	BC157824.1	UNC5A	0.0	99
3.4	BC157824.1	UNC5A	0.0	99
4.1	NM_000721.3	CACNAE1	0.0	100
5.6	AK313169.1	PRDX3	0.0	99
6.1	NM_007029.3	STMN2	0.0	99
6.4	NM_007029.3	STMN2	0.0	99
7.4	NM_007029.3	STMN2	0.0	99
8.1	AC009754.10	RP11-519C12	1e-114	76
11.1	NM_001328.2	CtBP1	0.0	99
12.2	AY207372.1	CCN1	4e-154	77

% ID: percent gene identity

Table 3.2: Yeast-2-hybrid assays between FANCC$^{1-306}$ and the identified clones

	pGBKFANCC$^{1-306}$		pGBKT7	
pACT2 clones	-TL	-TLHA	-TL	-TLHA
1.1 FANCC	+	-	+	-
2.1 UNC5A	+	+	+	-
3.4 UNC5A	+	+	+	-
4.1 CACNAE1	+	-	+	-
5.6 PRDX3	+	+	+	-
6.1 STMN2	+	+	+	-
6.4 STMN2	+	+	+	-
7.4 STMN2	+	+	+	-
8.1 RP11-519C12	+	-	+	-
11.1 CtBP1	+	+	+	-
12.2 CCN1	+	+	+	-

+ indicates growth on selective media

Figure 3.1: FANCC directly interacts with UNC5A in yeasts

		FANCC constructs				
UNC5A constructs		FANCC	FANCC$^{1-306}$	FANCC$^{307-558}$	FANCC$^{307-558\text{-}L554P}$	Empty
UNC5A-Y2H clone		+	+	-	+	-
UNC5A^{ICD}		-	+	-	-	-
UNC5A^{DD}		+	+	+	+	-
UNC5A$^{\Delta DD}$		-	+	-	-	-

Figure 3.2: FANCC interacts with UNC5A.

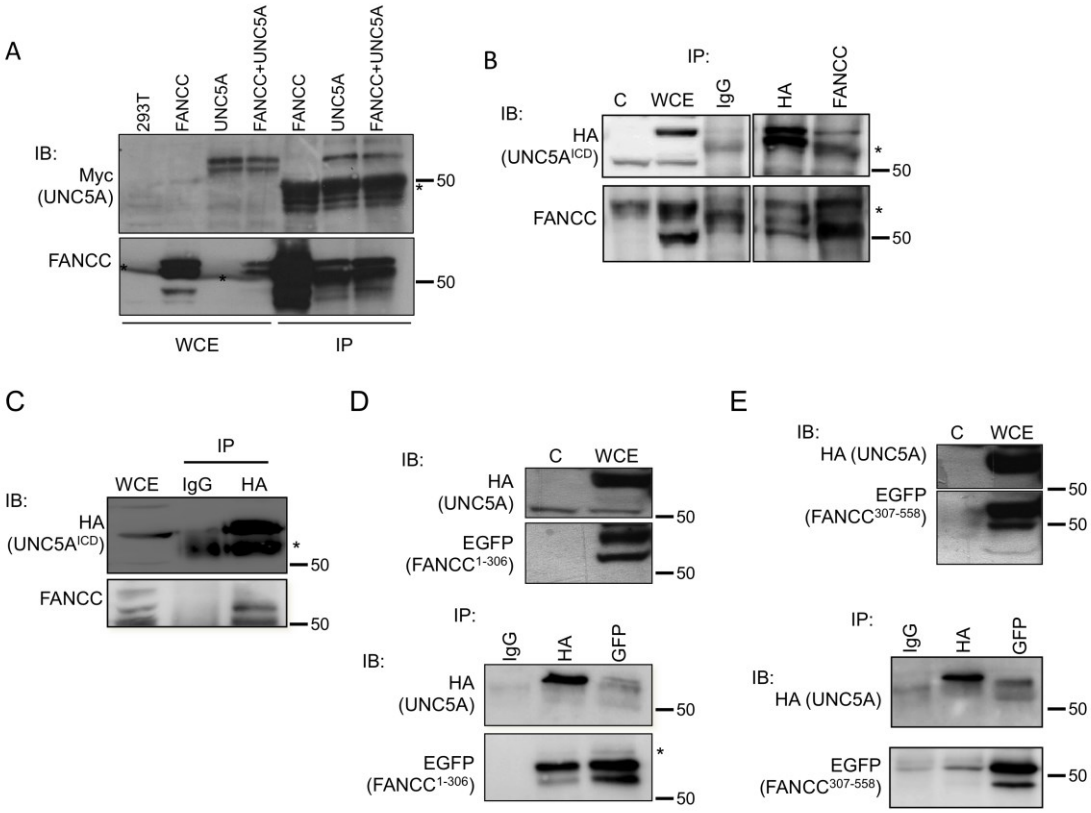


Figure 3.3: FANCC interacts with UNC5A$^{\Delta DD}$.

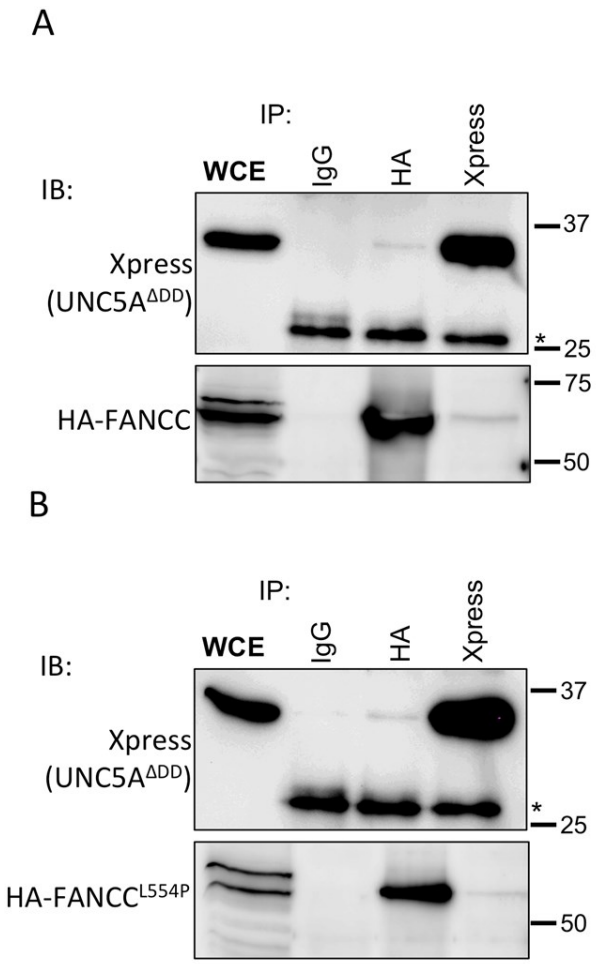


Figure 3.4: FANCC co-localizes with UNC5A in the cytoplasm.

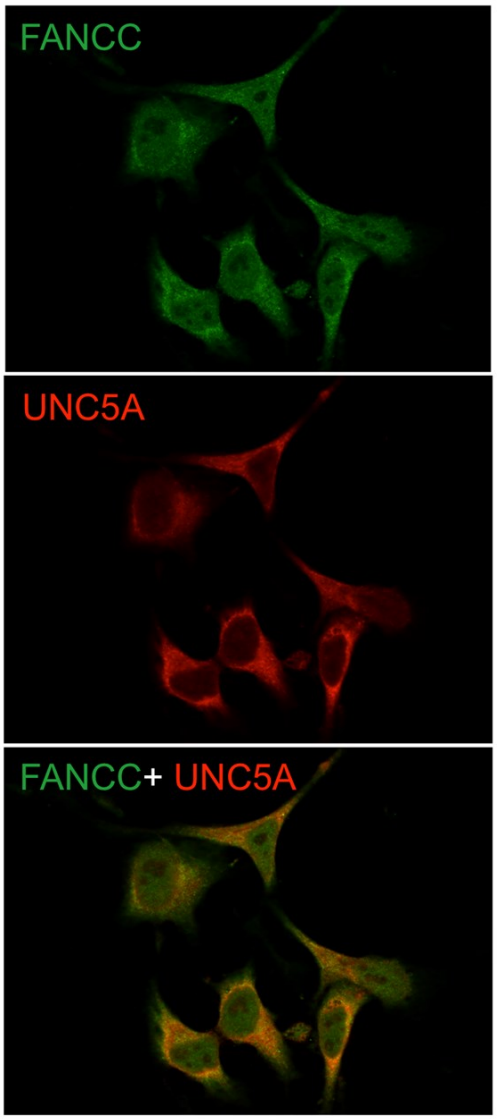


Figure 3.5: FANCC interferes with UNC5A-mediated apoptosis.

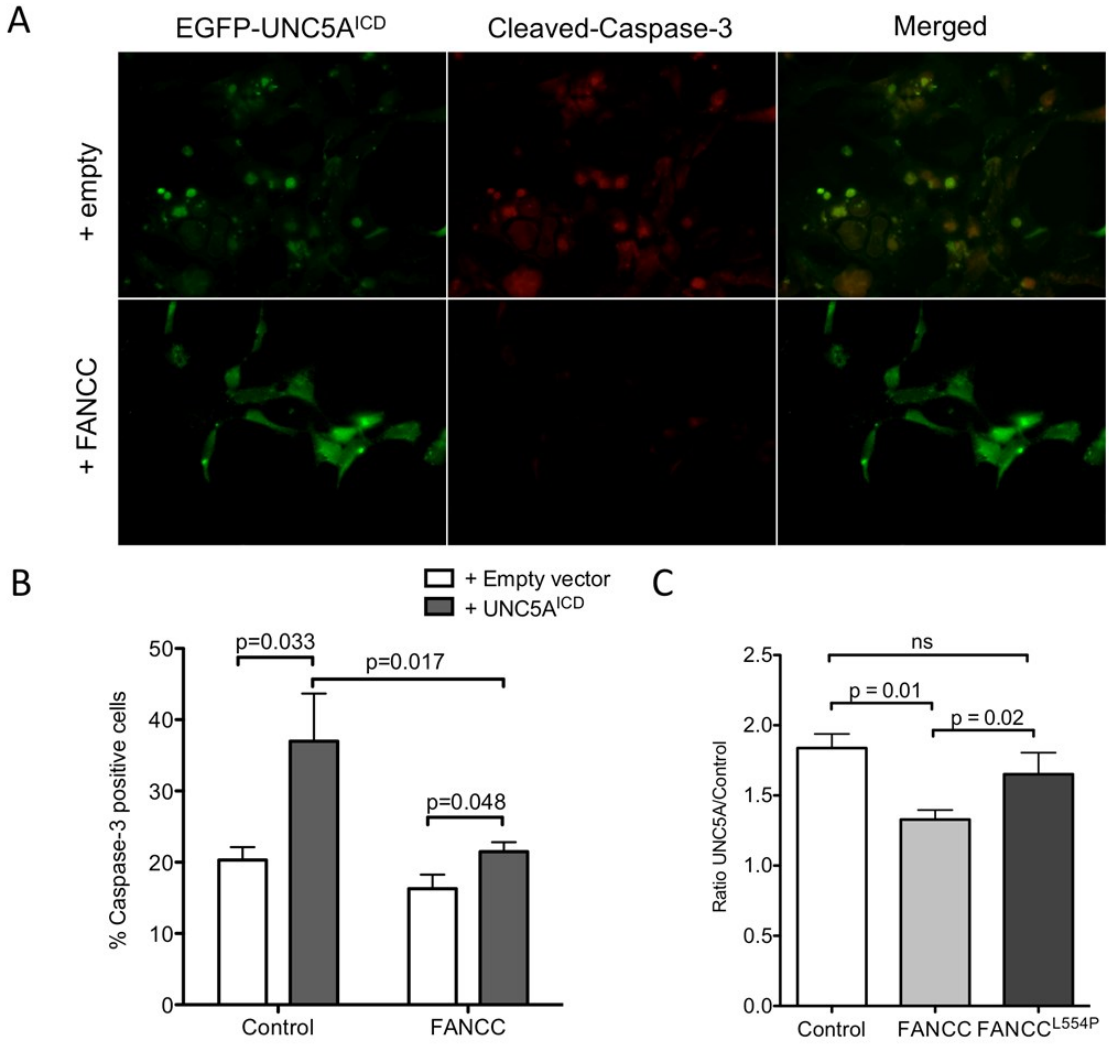


Figure 3.6: FANCC-depleted cells are sensitive to UNC5A-mediated apoptosis.

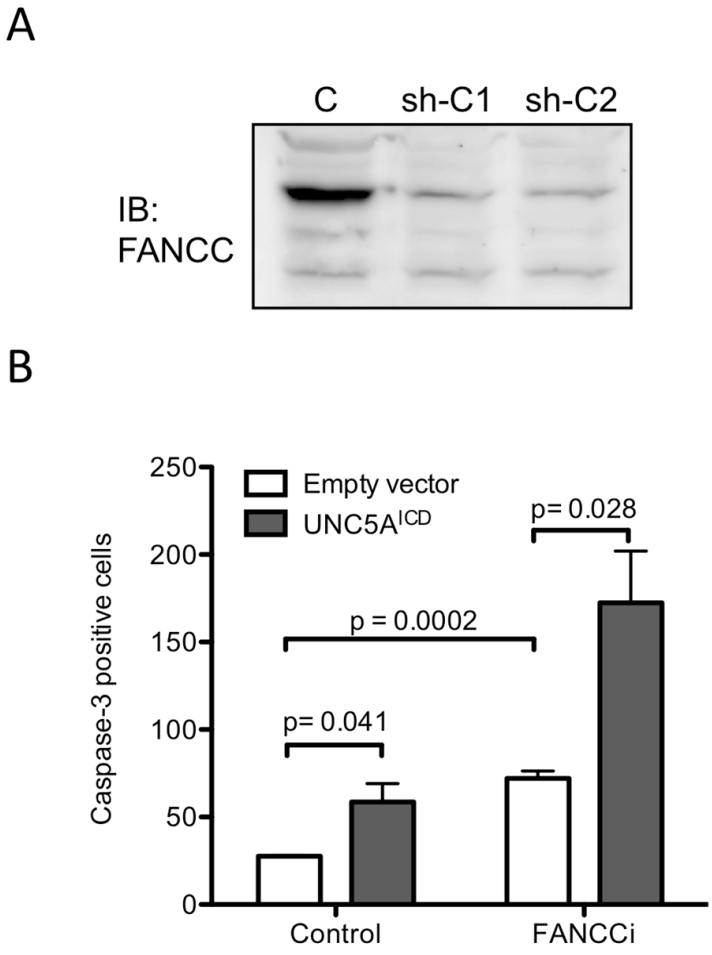
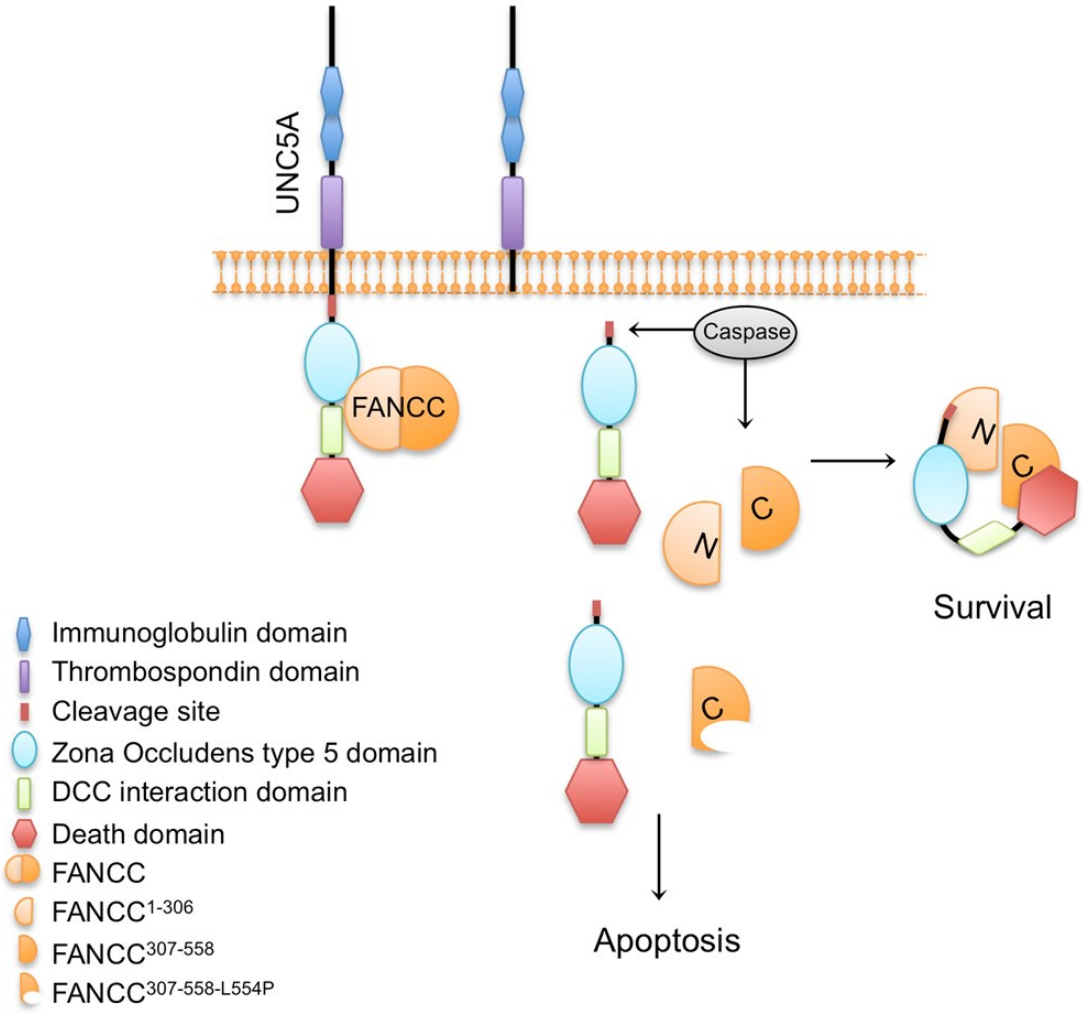


Figure 3.7: Schematic representation of FANCC interaction with UNC5A and possible role in apoptosis.



CHAPTER 4: RESULTS-ARTICLE II

FANCC increases UNC5A stability and co-localizes with UNC5A to induce neurite outgrowth

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In this paper, I (Fengfei Huang) performed all the experiments showed from Figure 4.1 to 4.4 and wrote the paper draft. My supervisor (Dr. Madeleine Carreau) did the correction.

4.1 Abstract

Fanconi anemia is a developmental deficiency disease, suggesting that FA proteins have functions other than those associated with DNA damage repair, for instance, in tissue homeostasis. FANCC, a predominantly cytoplasmic protein, has multiple functions including DNA damage signaling, oxygen radical metabolism, signal transduction, transcriptional regulation and apoptosis. Recently, FANCC has been found to be a direct partner of UNC5A and to delay UNC5A-mediated apoptosis. UNC5A is the primary receptor of the axon guidance molecule Netrin-1 and mediates a repulsive signal when bound to Netrin-1 during neural development and differentiation. The direct interaction of FANCC and UNC5A implies the existence of a function of FA protein during tissue development. Here we show that FANCC and UNC5A co-localize to regions of neurite outgrowth and that the absence of FANCC interferes with UNC5A-mediated neurite outgrowth. We also demonstrate that FANCC functions in positive mediation of UNC5A expression at both the gene and protein levels. Taken together, this evidence suggests that FANCC plays a crucial role in tissue development through the regulation of UNC5A-mediated functions.

4.2 Introduction

One of the major clinical manifestations of Fanconi anemia (FA) is developmental deficiency. Such deficiencies range from physically normal (approximately 25 to 40% of FA patients) to abnormalities so severe that they cause perinatal lethality [1-3]. Approximately half of children with FA have congenital skeletal

anomalies, frequently of the thumb (hypoplastic, duplicated, or absent) and the radius of the forearm (smaller or absent) [4-6]. In the most severe cases, developmental abnormalities simultaneously affect many organ systems, resulting in short stature, vertebral anomalies, anal atresia, cardiac abnormalities, tracheo-esophageal fistula, and/or renal anomalies, and may also include the central nervous system, the endocrine system and the gastrointestinal system [7-11]. The congenital malformations observed in FA probably represent the final outcome of inappropriate cell death during embryogenesis [12]. Although the role of FA proteins in DNA damage response signaling has been well characterized, the FA pathway cannot be used to explain the developmental deficiencies observed in the disease [13]. This suggests that FA proteins may have other functions such as the regulation of apoptosis or neuronal/tissue growth.

As a predominately cytoplasmic protein, the Fanconi anemia group C protein FANCC is a multifunctional protein that has roles in several cellular processes including DNA damage signaling, oxygen radical metabolism, signal transduction, transcriptional regulation and apoptosis [14]. In response to DNA crosslinking damage, FANCC associates with FANCE and translocates to the nucleus, where it cooperates with other components of the FA pathway to make up the FA core complex. During apoptosis, FANCC, which is considered a survival protein or anti-apoptotic regulator, undergoes proteolytic processing leading to the generation of cleaved protein fragments [15]. Currently, little is known about the molecular events leading to FANCC cleavage and its impact on downstream cellular signaling. We recently reported that FANCC directly interacts with uncoordinated-5A (UNC5A) and that it delays UNC5A-mediated

apoptosis.

UNC5A belongs to the UNC5 human transmembrane protein family, which includes the four homologous Netrin-1 receptors UNC5A, UNC5B, UNC5C and UNC5D. As a product of a homologue of the *C. elegans Unc-5* gene, UNC5 mediates a repulsive signal when bound to Netrin-1 during neural development and differentiation [16-19]. Recent research has shown that UNC5 protein expression is involved in tumorigenesis [20]. Loss or downregulated expression of the UNC5 gene is associated with various cancers and with tumor aggressiveness, supporting a role of UNC5 proteins as tumor suppressors [21]. In addition, UNC5 proteins have been proposed to function as 'dependence receptors', triggering apoptosis in the absence of their ligand and sending survival signals when bound to their ligand [22]. UNC5A has also been shown to promote apoptosis independently of Netrin-1, indicating the possibility of other functional ligands for this receptor [19, 23]. The intracellular ZU5 or death domain of UNC5A is essential for its effect on apoptosis. In a previous report, we showed that FANCC interacts directly with the UNC5A cytoplasmic death domain to delay UNC5A-mediated apoptosis [23-25]. In addition, gene expression studies in mice have shown that UNC5 receptors are expressed during early eye development, mammary bud formation, vascularization and limb development [26]. The expression of the Unc5A gene is restricted to the central nervous system (CNS), and FancC expression was detected in the mesenchyme of the spinal cord and in the actively dividing ependymal cell layer of the developing brain [27]. The overlapping expression patterns of FancC and Unc5A in the CNS suggest a possible role for UNC5A-FANCC during development and

neurogenesis.

In this study, we show that the FANCC and UNC5A proteins are both involved in neural differentiation. We also demonstrate that FANCC functions in the regulation of UNC5A expression and discuss how this regulation may explain the evidence that FANCC delays UNC5A-mediated apoptosis.

4.3 Materials and Methods

Plasmids and DNA constructs

Full-length FANCC was obtained using the expression plasmid pFAC3. The FANCC N-terminus, corresponding to the amino acid residues from the start codon to the cleavage site, and the FANCC C-terminus, corresponding to the cleaved FANCC C-terminus fragment, were cloned into plasmid pEGFP as previously described, yielding pEGFPFANCC$^{1-306}$ and pEGFPFANCC$^{307-558}$ [15]. The FANCE gene, which was obtained from the pREP4 expression plasmid, was subcloned into pCDNA3 as a fusion with a Myc expression protein. The intracellular domain of human UNC5A was cloned into the pCMVzeo vector in frame with a HA-tag (pCMVzeoUNC5A^{ICD}). The pCDNAHisXpres and pCDH-CMV-MCS lentiviral vectors were obtained as previously described [15]. The lentiviral vectors TRCN0000083368, TRCN0000083369 and TRCN0000083370 coding for shRNA against FANCC were obtained from Thermo Scientific. V2LHS-74072, V2LHS-74073, V3LHS-321475, V3LHS-321477, V3LHS-321480 coding for shRNA against FANCE and V2LHS-16512, V2LHS-16513, V2LHS-304038, V2LHS-304039, V2LHS-304040 coding for shRNA against UNC5A

were obtained from Thermo Scientific (ThermoFisher Scientific, Mississauga, ON).

Antibodies

The antibodies used in this study were as follows: anti-FANCE (Novus Biologicals); anti-UNC5A (Sigma-Aldrich); anti-FANCC (Novus Biologicals); 8F3 previously described [15] , a gift from Dr. M. Hoatlin (OSHU); anti-HA (12CA5, Roche Diagnostics, Indianapolis, IN); anti-cMyc (9E10, Santa Cruz Biotechnologies); anti-GFP (clone B2; Santa Cruz Biotechnologies); anti-GAPDH (1D4, Novus Biologicals); anti-goat (Calbiochem, San Diego, CA), anti-mouse and anti-rabbit (Santa Cruz Biotechnologies); donkey anti-rabbit Alexafluor 488 or Alexafluor 555 and anti-mouse Alexafluor 555 or Alexafluor 488 (Invitrogen, Burlington, ON). F-actin was labeled with Alexafluor 555 phalloidin (Life Technologies).

Cells, cell culture and transfection

HEK293T and HeLa cells were grown at 37°C in 5% CO_2 in DMEM medium supplemented with 10% FCS. SH-SY5Y cells were grown at 37°C, 5% CO_2 in a 1:1 mixture of DMEM and Ham's F12 Nutrient Mixture with 10% FCS. Cells were transfected by the calcium-phosphate transfection method or using lipofectamine 2000 (Invitrogen). For differentiation assays, SH-SY5Y cells were deprived of serum (0.5% FCS) for 48 hours or treated with retinoic acid (10 uM) for 48 hours prior to immunofluorescence staining. Cells were treated with recombinant human Netrin-1 (500 ng/ml, R&D systems) for 4 hours prior to immunofluorescence staining.

Western blotting analysis

For immunoblot analysis, total cell lysates were prepared in SDS loading buffer (50 mM Tris-HCL, 2% 2-mercaptoethanol, 2% sodium dodecyl sulfate). The samples were sonicated and/or boiled and subjected to electrophoresis on a 10% or 12% SDS-polyacrylamide gel. Proteins were electrotransferred onto a PVDF membrane (Amersham) and probed with antibodies as indicated in each figure.

Quantitative PCR

All animal procedures were performed according to protocols approved by the Animal Care Committee of Laval University, Québec, Canada. Brain cortex, hippocampus and bone marrow cells were obtained from 4- to 6-month-old *FancC*–/– and wild-type mice (C57BL/6J). Bone marrow cells were collected from femurs and tibias. Total RNA was isolated using the RNeasy Mini Kit RNA purification system according to the manufacturer's instructions (Qiagen). Reverse transcription was carried out with random hexamer primers (Ambion) using the SuperScript™II protocol as recommended by the manufacturer (Invitrogen). Quantitative PCR was performed with 100 nm each of the forward and reverse *Unc5A* or *Gapdh* primers using the ABI Prism 7000 Sequence Detection System and SYBRGREEN DNA binding dye (Invitrogen). Specific amplification was assessed based on the dissociation curve profile, and product sizes were verified by agarose 2% gel fractionation. The *Unc5A* gene expression profile was normalized to that of *Gapdh*.

Immunofluorescence microscopy

Localization analyses of FANCC and UNC5A were performed by immunofluorescence microscopy as follows. HEK293T, HeLa or SH SY5Y cells were grown for 24 hours under the appropriate culture conditions on glass coverslips (12-mm diameter) prior to fixing with 4% paraformaldehyde in PBS for 20 minutes at room temperature. Following permeabilization for 15 minutes at room temperature with 0.3% Triton X-100 in PBS, the fixed cells were incubated with specific primary antibodies (anti-FANCC 8F3 and anti-UNC5A) and subsequently with secondary antibodies (goat anti-mouse Alexafluor-488; goat anti-rabbit Alexafluor-546 or donkey anti-rabbit Alexafluor-555) at the appropriate dilutions in PBS with 10% horse serum. The cells were washed 3 times with PBS, and nuclei were labeled with DAPI. Images were acquired using a Nikon E800 fluorescent microscope equipped with a C1 confocal system (Nikon Canada).

Statistical analyses

Data are expressed as mean ± standard error of the mean (SEMs). Statistical analyses were performed using GraphPad Prism software (version 5.0b; GraphPad Software Inc., San Diego, CA), and paired and unpaired two-tailed Student's t-tests were used to compare the means. P values less than 0.05 were considered significant.

4.4 Results

FANCC$^{307-558}$ increases the stability of UNC5A protein

We observed increased expression of UNC5A when the latter protein was co-expressed with FANCC constructs. To test whether increased levels of UNC5A correlate with FANCC protein levels, we performed studies in which UNC5A was co-expressed with various amounts of FANCC or its cleaved products, $FANCC^{1-306}$ and $FANCC^{307-558}$. Because FANCE has been shown to increase FANCC protein stability [28] as well as the stability of the FANCC C-terminal cleavage product, ($FANCC^{307-558}$ and data not shown), FANCC constructs were expressed concomitantly with a FANCE coding vector. HEK293T cells transfected with increasing amounts of FANCC, FANCE or $UNC5A^{ICD}$ were compared to cells expressing equimolar amounts of each coding vector. The results showed that co-expression of $UNC5A^{ICD}$ and FANCC led to a slight increase in UNC5A protein levels compared to FANCE expression (Figure 1A), whereas co-expression of $UNC5A^{ICD}$ with ten times the amount of FANCC or FANCE did not significantly alter the levels of $UNC5A^{ICD}$ (Figure 1B and 1E). As expected, FANCE expression increased the stability of FANCC, as previously reported [28]. Co-expression of $FANCC^{1-306}$ with $UNC5A^{ICD}$ had no effect on UNC5A protein levels; however, transfection of the cells with ten times the amount of the FANCE expression vector led to a slight increase in $FANCC^{1-306}$ and decreased $UNC5A^{ICD}$ protein levels compared to cells transfected with equimolar amounts of each coding vector (Figure 1C and 1E). Further analysis showed that these changes in UNC5A protein expression were not statistically significant (Figure 1E).

We next evaluated the impact of $FANCC^{307-558}$ on UNC5A protein levels. The results of these experiments showed that cells transfected with ten times the amount of

FANCC$^{307\text{-}558}$ expression vector showed a dramatic increase in UNC5A^{ICD} protein levels, up to twelve times the expected level (Figure 1D-E). Overexpression of FANCE had no effect on UNC5A^{ICD} protein levels, but it resulted in elevated FANCC$^{307\text{-}558}$ protein levels compared to cells transfected with equimolar amounts of each coding vector (Figure 1D). These results suggest that the C-terminal cleavage product of FANCC, FANCC$^{307\text{-}558}$, positively impacts UNC5A^{ICD} protein stability.

FANCC and FANCE upregulate UNC5A mRNA expression

We next investigated whether the level of UNC5A mRNA in cells is regulated by FANCC. Because FANCC and FANCE depend on each other for translocation to the nucleus and FANCE has been shown to increase the stability of FANCC, the possible function of FANCE in regulating UNC5A mRNA expression was also investigated. We transfected HEK293T cells with FANCC, FANCE or UNC5A^{ICD}, extracted endogenous mRNA of UNC5A and compared the cDNA level to that in cells expressing equimolar amounts of each empty coding vector. The results showed that both FANCC and FANCE significantly upregulate the expression of endogenous mRNA of UNC5A (Figure 2A-D). As expected, the endogenous expression of FANCC and FANCE is stable; the levels of FANCC mRNA do not affect those of FANCE mRNA, and vice versa (Figure 2A). These results suggest that both FANCC and FANCE function to regulate the transcription of UNC5A and may positively mediate the protein level of UNC5A as our findings show that FANCC$^{307\text{-}558}$ positively impacts UNC5A^{ICD} protein stability. Further experiments are needed to directly investigate FANCC$^{307\text{-}558}$ function

on regulating the transcription of UNC5A^{ICD} and the mechanism under these regulations between FANCC, FANCE and UNC5A.

UNC5A expression is reduced in FANCC knockout mice

UNC5A–D gene expression has been examined in 240 tumors and compared to expression in corresponding normal tissues; it was shown to be markedly downregulated in most of the tested tumors. Reduction in UNC5A–D gene expression was observed in 93% of colorectal tumors, 88% of ovarian tumors, 81% of renal tumors, 74% of lung tumors, 68% of stomach tumors, 49% of breast tumors and 48% of uterine tumors [29]. Our previous results show that FANCC stabilizes UNC5A expression at both the protein and gene levels. These results induced us to measure whether UNC5A expression is different in the FANCC knockout mouse, in which interaction between FANCC and UNC5A does not occur. Using western blotting and q-PCR, we compared UNC5A protein and mRNA levels in FANCC knockout and wild-type mice. The results showed that UNC5A protein is significantly downregulated in the cerebral cortex of FANCC knockout mice (Figure 3A-B). The q-PCR results show that the UNC5A gene is downregulated in bone marrow (Figure 3C), whereas in cerebral cortex and hippocampus, its expression is not significantly different from that in wild-type mice (Figure 3D). As noted before, UNC5 proteins are frequently inactivated in a number of cancers, a finding that is consistent with our result that UNC5A gene expression is downregulated in FANCC-depleted bone marrow, a condition that might promote leukemia development. Our inconsistent findings regarding UNC5A gene and protein expression in the cerebral cortex suggest other factors in addition to FANCC regulate

UNC5A protein expression.

FANCC and UNC5A are required for neuronal differentiation

Although the role of FA proteins is generally thought to be associated with hematopoiesis, the work of Sii-Felice *et al.* has established that FA proteins are required for the development and survival of neural progenitor cells [30]. In addition, gene expression studies have shown that both *FancA* and *FancC* are highly expressed in the developing brain, specifically in the intermediate zone, which contains migrating neurons [31-33]. Because UNC5 proteins, including UNC5A, have been associated with axonal guidance, neuronal differentiation and survival [34], we hypothesized that FANCC, in association with UNC5A, may be involved in these processes. To test this hypothesis, we used SH-SY5Y neuroblastoma-derived cells, a model for studying neurons. SH-SY5Y cells were depleted of UNC5A or FANCC and subsequently induced to differentiate by serum deprivation. Upon serum deprivation, the cells showed morphological changes and neurite outgrowth characteristic of differentiation, consistent with previous reports [35, 36]. However, depletion of UNC5A or FANCC in SH-SY5Y cells resulted in a marked reduction in neurite outgrowth following serum-induced differentiation (Figure 4A). We also observed that cells depleted of UNC5A or FANCC showed decreased cell growth and increased cell death (Figure 4A and data not shown). Labeling of F-actin with phalloidin conjugate confirmed the reduced branching and outgrowth of neurites in cells depleted of either FANCC or UNC5A (Figure 4B). Consistent with the requirement for these proteins in neurite outgrowth, both UNC5A and FANCC co-localized to neuronal growth cones following

differentiation induced by serum deprivation (Figure 4D). In addition, the presence of recombinant Netrin-1 during the differentiation process resulted in strong co-labeling of UNC5A with FANCC at the ends of neurites (Figure 4E-F). These results imply that FANCC plays a role in the transmission of cellular signals via UNC5A during neurite outgrowth.

4.5 Discussion

Fanconi anemia is a developmental deficiency disease, and FA patients display diverse developmental abnormalities. In adult Fanconi mice, reduced proliferation of neural progenitor cells related to apoptosis and accentuated neural stem cell exhaustion with aging were observed [37, 38]. In addition, embryonic and adult Fanconi neural stem cells showed a reduced capacity to self-renew in vitro. Taken together, these clues suggest that FA proteins are likely to possess functions related to tissue homeostasis that differ from their known function in DNA damage repair; however, a clear link remains elusive. Recently, FANCC has been shown to function as a direct partner of UNC5A and to delay UNC5A-mediated apoptosis [37, 38]. However, the functional significance of the association between FANCC and UNC5A is unknown. As the receptor of Netrin-1, UNC5A was shown to mediate a repulsive signal when coexist with DCC receptor and binding to Netrin-1 during neural development and differentiation [16-19, 39]. UNC5 receptors have also been shown to control the morphogenesis of non-neuronal tissues, as well as to guide migrating cells during angiogenesis, mammary gland formation and lung development [40-42]. Studies of the expression of *Unc5* genes during mouse

development showed that UNC5 homologues are highly expressed in developing limbs [26, 43, 44]. Similarly, during development FA genes are expressed primarily in cells of mesenchymal origin that give rise to forelimb and hindlimb tissues [31-33]. Taken together, this evidence suggests that FANCC may play a role in tissue morphogenesis through its interaction with UNC5A. This paper provides direct evidence that the FANCC-UNC5A complex functions in neurogenesis by showing that FANCC and UNC5A co-localize to neurite-type extensions of neuronal cells and that there is strong immunoreactive staining for both proteins at the synapses of neurites. In addition, we observed that FANCC requires UNC5A to promote neuronal outgrowth. Recent research has shown that Netrins regulate synaptogenesis in *C. elegans* either by promoting presynaptic assembly through the DCC/UNC-40 receptor [45, 46] or by preventing inappropriate localization of presynaptic components through the UNC-5 receptor [47, 48]. The data presented here suggest that FANCC is involved in the mediation of tissue homeostasis through interaction with UNC5A.

Loss or downregulation of UNC5A gene expression is related to the occurrence of various cancers, including colorectal, breast, ovarian, uterine, stomach, lung, and kidney cancers. This evidence supports a role for UNC5 proteins as tumor suppressors [21]. However, the mechanism of this downregulation of the UNC5 gene is unknown. Our current data show that the C-terminal cleavage product of FANCC, $FANCC^{307-558}$, positively impacts $UNC5A^{ICD}$ protein stability. Both FANCC and FANCE significantly upregulate endogenous mRNA expression of UNC5A. In addition, expression of UNC5A at both the protein and mRNA level is negatively mediated in brain cortex and

bone marrow tissue in the *FANCC-/-* mice compared to wild-type mice. These results are consistent with the previous finding that the UNC5A gene is inactive in various cancers and suggest that FANCC is a critical modulator of UNC5A expression. Evidence has shown that the abnormal apoptosis that occurs in FA is likely related to increased inactivation of p53 during development [49]. UNC5A has been identified as a direct p53 target gene and is involved in the p53-dependent apoptotic response to DNA damage [50]. Thus, FANCC may be associated with the p53 signaling pathway through its interaction with UNC5A.

This paper provides direct evidence that FANCC, together with UNC5A, may be responsible for signal transduction events in the cell that are required for tissue morphogenesis. In view of the fact that FA patients show various developmental abnormalities and that cells derived from FA patients are hypersensitive to apoptotic cues, we propose that FANCC plays a key role either by delaying UNC5A-mediated apoptosis or by positively impacting the expression of UNC5A. In the FANCC-deficient condition, one subtype of FA in which the FANCC-UNC5A interaction is disrupted, the resulting dysregulation of the UNC5A signaling pathway would lead to developmental defects.

4.6 Acknowledgment

We are grateful to Dr. Maureen Hoatlin (OHSU) for providing 8F3 monoclonal anti-FANCC antibodies.

4.7 Reference

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4.8 Figure legends

Figure 4.1: FANCC$^{307-558}$ increases UNC5A protein stability.

HEK293T cells were transfected with UNC5A^{ICD}, FANCE and FANCC constructs expressing full-length FANCC (in *A-B*), FANCC$^{1-306}$ (in *C*) or FANCC$^{307-558}$ (in *D*). Constructs were transfected at a molecular ratio of 1:1:1 or with 10 times the molar amount of FANCE, FANCC or UNC5A^{ICD} as indicated in the figure. The total amount of transfected plasmid was equalized for all strategies with control empty vectors. Representative immunoblots performed with anti-HA (UNC5A^{ICD}), anti-FANCC, anti-FANCE or anti-GAPDH antibodies are shown; * indicates nonspecific bands. (E) The bar graphs represent the mean fold change ± SEM of UNC5A protein expression normalized to GAPDH compared to 1:1:1 transfection controls in at least 4 separate experiments. * $p< 0.05$; ** $p<0.005$.

Figure 4.2: FANCC and FANCE upregulate mRNA expression of UNC5A.

(A) HEK293T cells were transiently transfected with UNC5A^{ICD}, FANCC and FANCE constructs expressing full-length FANCC and FANCE separately or with equimolar amounts of corresponding control empty vector. The representative RT-PCR shown was performed with specific oligos and amplified parts of UNC5A, FANCC and FANCE.

(B-D) The bar graphs represent the mean fold change ± SEM of UNC5A, FANCC and FANCE cDNA expression normalized to GAPDH compared to empty vector controls in 3 separate experiments. * $p< 0.05$.

Figure 4.3: Comparison of UNC5A proteins and genes in *FANCC* -/- and wild-type mice.

(A) The brain cortex protein of *FANCC*-/- mice (n=7) and wild-type mice (n=6) was extracted, and UNC5A protein expression was detected by western blotting. Tubulin expression was used as a protein quantity control.

(B) The bar graphs shown represent the mean fold change ± SEM of UNC5A protein expression in *FANCC*-/- and wild-type mice normalized to tubulin protein expression from 2 separate experiments. * $P < 0.05$.

(C) Total bone marrow mRNA was extracted from *FANCC*-/- mice (n=10) and wild-type animals (n=10) and used in q-PCR experiments. The bar graphs represent the mean fold change ± SEM of UNC5A cDNA expression in the wild-type group normalized to that of the *FANCC*-/- group in 3 separate experiments. * $P < 0.05$.

(D) Total mRNA from brain cortex and hippocampus of *FANCC*-/- mice (n=3) and wild-type animals (n=4) was extracted and used in q-PCR experiments. The bar graphs represent the mean fold change ± SEM of UNC5A cDNA expression in the wild-type group normalized to that of the *FANCC*-/- group in 2 separate experiments.

Figure 4.4: FANCC and UNC5A are required for neurite outgrowth and co-localize to the extremities of neuritis.

(A) Representative microscopic images of SH-SY5Y cells stably transduced with shRNA against FANCC, FANCE, UNC5A, or control noncoding scrambled shRNA (control) vectors.

(B) Cells induced to differentiate in the presence of retinoic acid (RA) as a function of time. Representative microscopic images of 3 separate experiments performed in duplicate are shown at 40X magnification.

(C) Western blots showing the depletion of FANCC, FANCE and UNC5A in SH-SY5Y cells.

(D) UNC5A- and FANCC-depleted SH-SY5Y cells (UNC5Ai- and FANCCi-treated, respectively) were induced to differentiate with RA and labeled with anti-UNC5A (blue) and anti-FANCC (green) antibodies and phalloidin conjugates (red). The labeled cells were visualized by confocal fluorescence microscopy at 100X magnification using a Nikon E800 microscope equipped with a C1 confocal system.

(E) SH-SY5Y cells differentiated by retinoic acid treatment were labeled with antibodies against FANCC (green) and UNC5A (red).

(F) SH-SY5Y cells were incubated with recombinant Netrin-1 (500 ng/ml) prior to analysis. Labeled cells were visualized by confocal fluorescence microscopy at 60X and 100X magnification using a Nikon E800 microscope equipped with a C1 confocal system.

Figure 4.1: FANCC$^{307-558}$ increases UNC5A protein stability

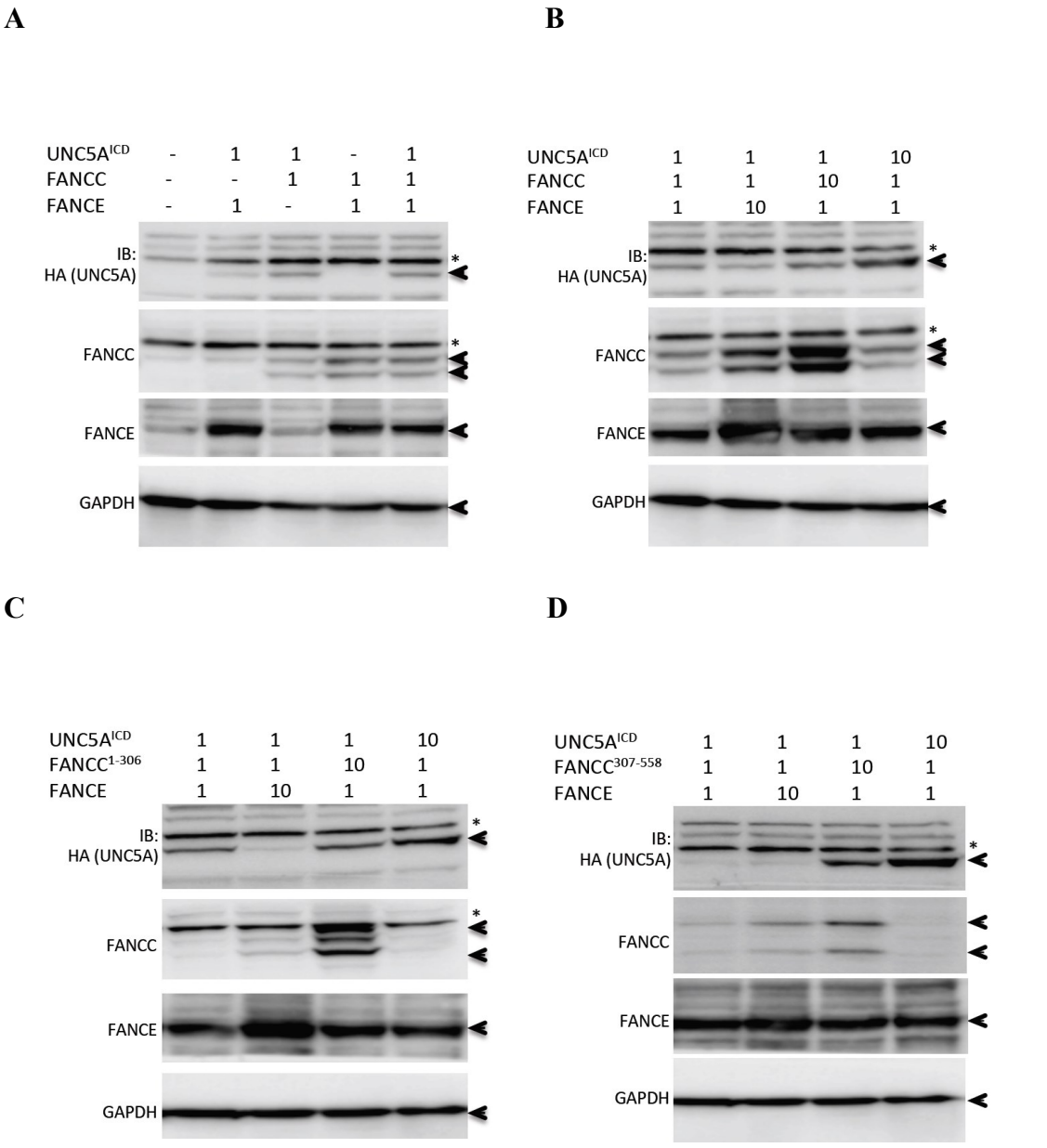
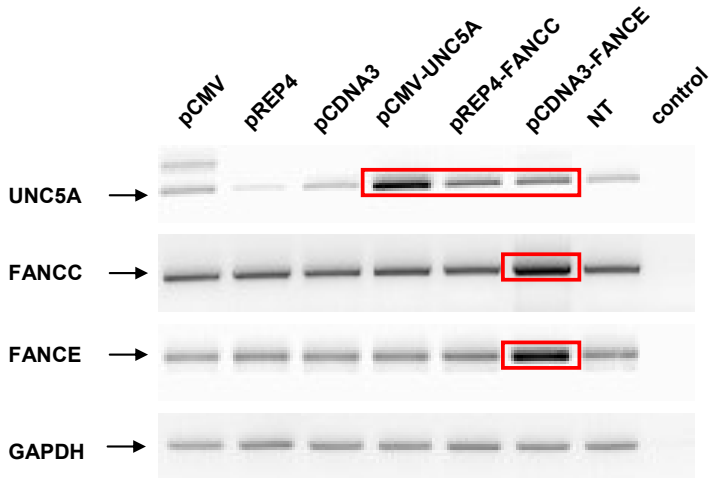
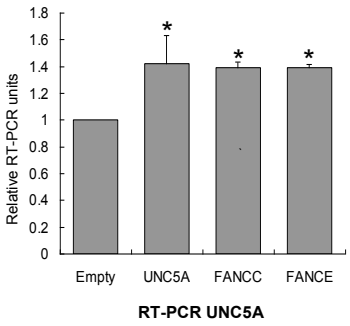


Figure 4.2: FANCC and FANCE upregulate mRNA expression of UNC5A

A



B



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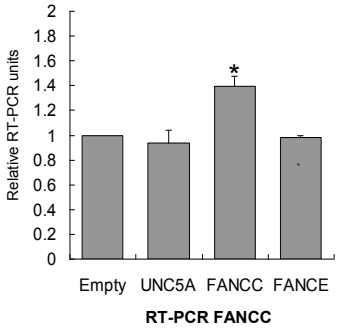

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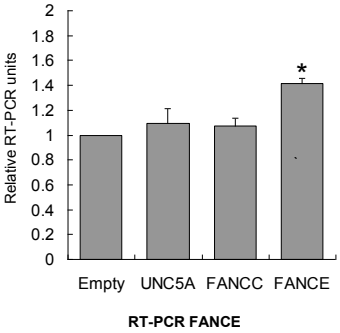
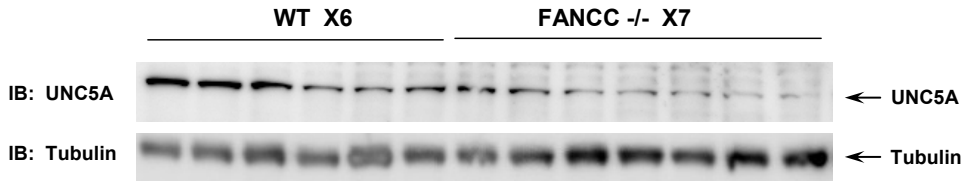
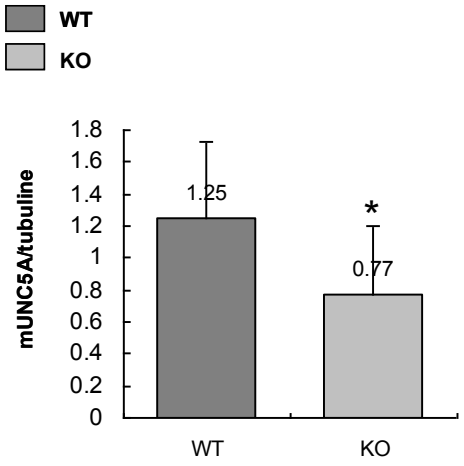


Figure 4.3: Comparison of UNC5A proteins and genes in *FANCC* -/- and wild-type mice

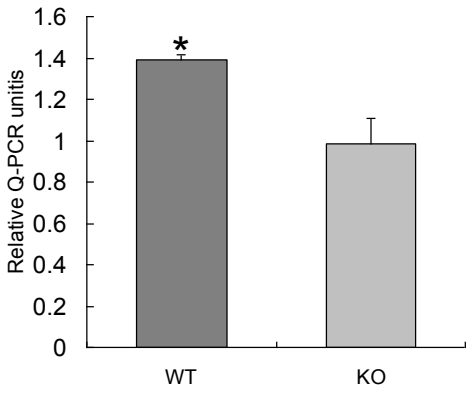
A. Brain



B. Brain



C. Bone marrow



D. Brain

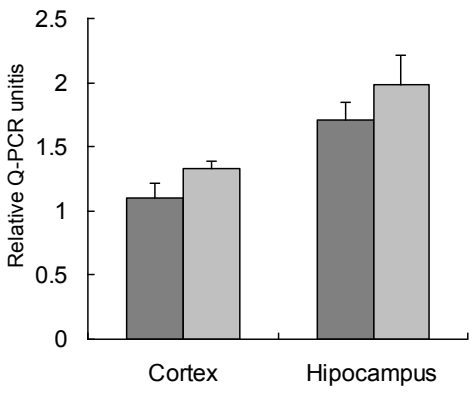
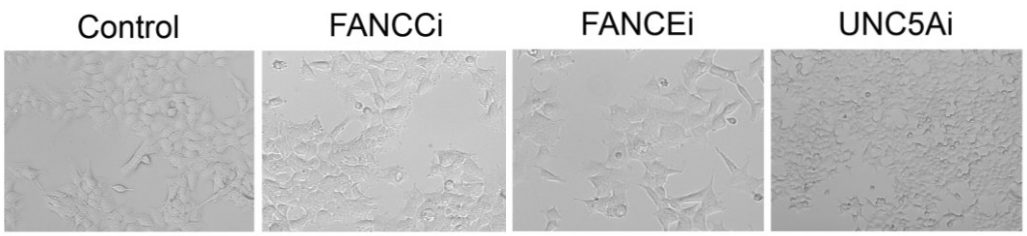


Figure 4.4: FANCC and UNC5A are required for neurite outgrowth and co-localize to the extremities of neurites

A



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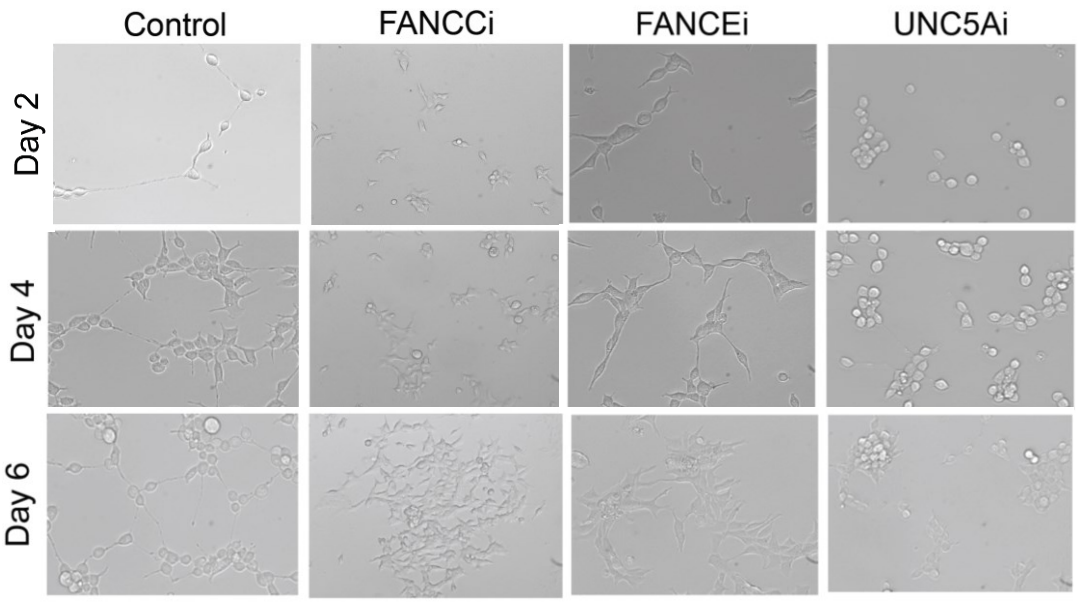

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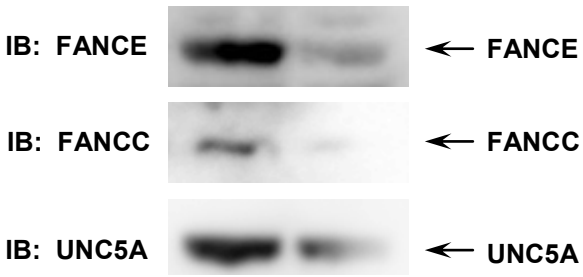
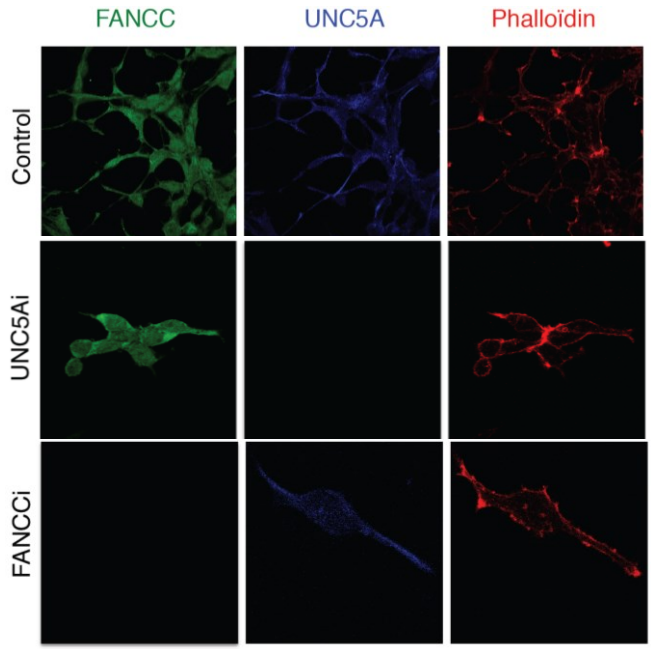
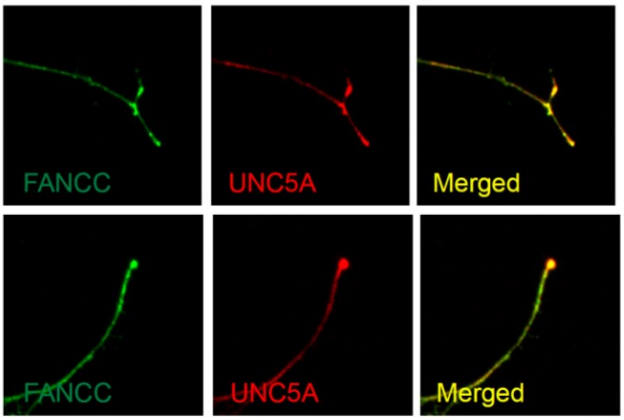


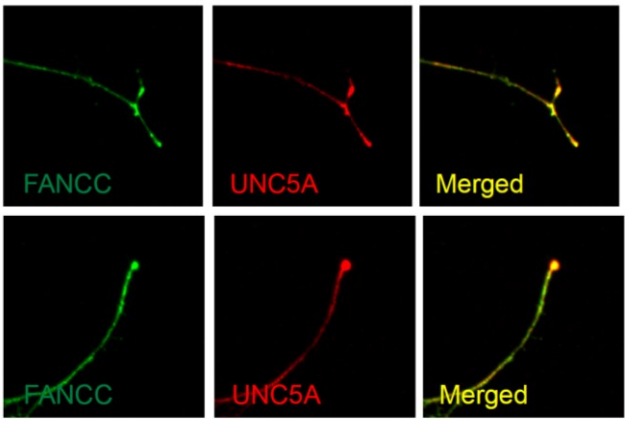
D


FANCC
UNC5A
Phalloïdin
Control
UNC5Ai
FANCCi

E


FANCC
UNC5A
Merged
FANCC
UNC5A
Merged

F


FANCC
UNC5A
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FANCC
UNC5A
Merged

CHAPTER 5: DISCUSSION AND CONCLUSIONS

5.1 The role of FANCC-UNC5A interaction during apoptosis

The clinical manifestations of FA, which include life-threatening hematologic deficiency and predisposition to multiple neoplasia, imply that the impairment of several signaling pathways, possibly including abnormal apoptosis signaling, may contribute to the disease mechanism.

Unlike other known FA proteins, FANCC predominately exists in cytoplasm, which implies that FANCC might have other functions in addition to DNA damage signaling. In fact, FANCC protein has been shown to have multiple functions associated with oxygen radical metabolism, signal transduction, and transcriptional regulation. In addition, FANCC is a well-known apoptosis suppressor protein; FANCC-depleted cells or patient-derived cells with *FANCC* mutations show increased apoptosis upon exposure to various cellular stressors, whereas FANCC overexpression delays the onset of apoptosis [232]. During apoptosis, FANCC is regulated by caspase-mediated cleavage, which inactivates its ability to suppress apoptosis [118]. To better understand the impact of FANCC in apoptosis regulation, a yeast two-hybrid screen was performed and the dependence receptor UNC5A was identified as one of the possible interaction partners of FANCC [233].

UNC5A is the receptor of the axon guidance molecule Netrin-1. In addition to its function as a repulsive mediator during neural development, UNC5A has also been proposed as one of several “dependence receptors” that trigger apoptosis in the absence of their ligands [197]. The ZU-5 region and the Death domain in the C-terminal region of UNC5A are required to bind NRAGE, which is crucial for UNC5A mediation of apoptosis [199, 203].

The results presented in chapter 3 of this thesis demonstrate that UNC5A is a novel interaction partner of FANCC. In fact, UNC5A also interacts with other FA protein members (supplementary figure 1). As shown by yeast-two hybrid analysis, $UNC5A^{ICD}$, $UNC5A^{C\text{-}ter}$ and the clone BC157824.1 all interact directly with FANCF, FANCG and FANCL, whereas only clone BC157824.1 interacts with FANCA. In cells, both FANCA and FANCE show slight interaction with $UNC5A^{ICD}$. Together with the results showing that UNC5A interacts with FANCC, UNC5A might be a potential partner of the FA core complex, and these interactions are likely to occur via its intracellular portion. The exact binding region and the influence of these interactions on the FA pathway await further investigation.

Our results show that FANCC is a positive regulator to UNC5A-mediated apoptosis. Under conditions of FANCC overexpression, apoptosis is decreased, whereas the absence of functional FANCC protein increases UNC5A-mediated apoptosis. In fact, this interaction occurs through the ZU-5 region and Death domain of UNC5A, and this interaction may interfere with its binding to apoptosis-promoting factors such as MAGED1. In addition, UNC5A has been identified as a direct p53 target gene and shown to be involved in the p53-dependent apoptotic response to DNA damage [213]. Through its interaction with UNC5A, FANCC may be associated to p53-induced apoptosis pathway. Thus, FANCC may regulate cellular sensitivity to UNC5A-mediated apoptosis and thereby act as a sensor of cellular stress and a switch between apoptosis and survival.

5.2 The role of FANCC-UNC5A interaction during development

Fanconi anemia is a developmental deficiency disease, and FA patients display diverse developmental abnormalities. However, little is known about the molecular

mechanisms underlying these abnormal congenital clinical phenotypes. UNC5A, as the receptor of Netrin-1, has been shown to be a mediator for repulsive signaling in the neural development and differentiation [198, 227-229] and has also been shown to control the morphogenesis of non-neuronal tissues during angiogenesis, mammary gland formation and lung development [195, 234, 235]. Together with its role as a dependence receptor in apoptosis, UNC5A is considered to be a crucial physiological regulator of tissue size and shape. In FA, the FANCC-UNC5A interaction is interrupted and UNC5A's apoptotic signal is dysregulated, which could lead to developmental defects similar to those observed in FA patients.

Study of the expression of UNC5 during development has shown that UNC5 homologs are highly expressed in developing limbs [201]. Similarly, FA genes are expressed primarily in cells of mesenchymal origin that give rise to forelimb and hind limb tissues [120, 121, 236]. Consequently, defective FA genes result in limb malformations such as absence or underdevelopment of thumbs or short or hypoplastic radii as observed in FA patients [10]. The results presented in chapter 4 of this thesis provide direct evidence that FANCC and UNC5A function as a complex in neurogenesis by demonstrating that the two proteins co-localize at the synapses of neurites. In addition, we also observed that the promotion of neuronal outgrowth by UNC5A requires FANCC. These data suggest that FANCC is involved in the regulation of synaptogenesis through its interaction with UNC5A [191, 192].

Loss of UNC5 gene expression has been associated with various cancers, and this evidence supports a role for UNC5 proteins as tumor suppressors [237237] . However, the molecular events surrounding the downregulation of the UNC5 gene are elusive. Our data show that both FANCC and FANCE upregulate UNC5A gene expression and that the C-terminal cleavage product of FANCC, $FANCC^{307-558}$, positively impacts

UNC5A^{ICD} protein stability. These results are consistent with other data we obtained by Q-PCR and Western blotting showing that both protein and gene expression of UNC5A are downregulated in the brain cortex and bone marrow tissue of *FANCC-/-* mice compared to wild-type animals. Based on this finding and on our previous results, we propose that FANCC plays a key role in tissue morphogenesis either by delaying UNC5A-mediated apoptosis or by positively impacting the expression of UNC5A. Under FANCC-deficient conditions, dysregulation of the UNC5A signaling pathway would then lead to developmental defects.

Our results provide direct evidence that the interaction of FANCC with UNC5A may be responsible for signal transduction events in the cell that are required for tissue morphogenesis. The newly discovered interaction between FANCC and UNC5A provides a novel clue to a better understanding of the role of FA proteins in development.

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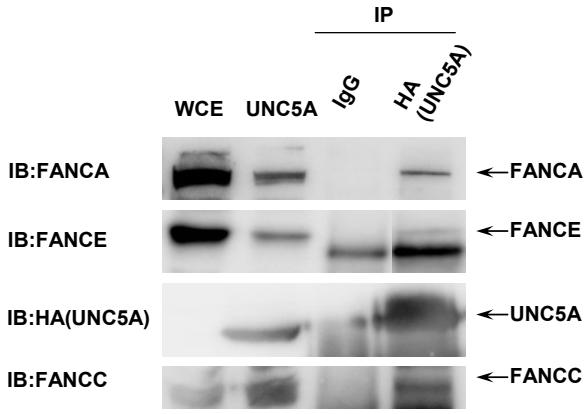
SUPPLEMENTARY RESULTS

A.

UNC5A constructs	FA members						
	FANCA	FANCD2	FANCE	FANCF	FANCG	FANCL	Empty
ZU-5 DEATH	–	–	–	+	+	+	–
DEATH	+	–	–	+	+	+	–
DEATH	–	–	–	+	+	+	–

B.



Supplementary figure S 1: UNC5A interacts directly with FA member proteins.

(A) Yeast two-hybrid assays with UNC5A constructs including the intracellular domain, a Y2H clone coding for the death domain (DEATH) and part of intron 14 (shown in blue) and UNC5A C-terminal end and FANCA, FANCD2, FANCDE, FANCF, FANCG, FANCL. A positive interaction is indicated as +. Negative controls were performed with empty vectors (Empty), and positive controls were performed with pGBKT7/p53 and pGADT7/SV40 T-antigen from Clontech (data not shown). Yeast two-hybrid assays were performed at least three times (independent transformations) with 3 to 4 clones each.

(B) Co-immunoprecipitations were performed in HEK293T cells transiently transfected with HA-tagged UNC5A^{ICD}. Immunoprecipitations (IP) were performed with anti-HA

antibody. Negative IP were performed with anti-mouse (M) antibodies. Endogenous FANCA, FANCE and FANCC were immunoblotted with the indicated antibodies.