Suppression of the Ubiquitin Ligase Function of FBXW7 Accelerates Metastatic Progression of Pancreatic Ductal Adenocarcinoma

by

Grissel Cervantes Jaramillo

B.S. Biochemistry and Molecular Biology
University of Miami, 2017

Submitted to the Harvard-MIT Program in Health Sciences and Technology
In Partial Fulfillment of the Requirements for the Degree of

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Authored by: Grissel Cervantes Jaramillo
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Certified by: Tyler E. Jacks PhD
David H. Koch Professor of Biology
Thesis Supervisor

Accepted by: Collin M. Stultz, MD, PhD
Director, Harvard-MIT Program in Health Sciences and Technology
Robert H. Rubin Professor in Medical Engineering and Science/Professor of Electrical Engineering and Computer Science.

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ABSTRACT

Pancreatic ductal adenocarcinoma (PDAC) is the most lethal common malignancy because it is usually diagnosed at an advanced/metastatic stage. Dysregulation of protein stability and degradation has been associated with uncontrolled proliferation and genomic instability, promoting cancer progression to metastasis. One of the major regulators of protein degradation is the tumor suppressor FBXW7, a substrate recognition domain of the SCF E3 ubiquitin ligase, frequently dysregulated in many cancers.

The function and clinical significance of FBXW7 in pancreatic cancer has been studied in some detail. Pancreatic cancer patients with low FBXW7 expression levels have poor probability of survival compared to patients with high FBXW7 expression levels. Furthermore, Fbxw7 mutations and loss cooperate with $Kras^{G12D}$ to accelerate PDAC formation with a high frequency, showing that Fbxw7 is an important tumor suppressor in Kras-driven pancreatic cancer. However, studies on the impact of Fbxw7 expression and its substrates in pancreatic cancer progression to metastasis remains poorly understood.

Here, we demonstrate that *Fbxw7* loss accelerates progression and metastatic potential of pancreatic cancer in $Kras^{G12D/+}$; $Trp53^{-/-}$ PDAC models, in immunocompromised and immunocompetent hosts. We explore the impact of different Fbxw7 mutants in tumorigenesis, where the hotspot mutant R465 recapitulates the phenotype seen in complete loss-of-function of Fbxw7. Finally, we looked at global proteomic changes when Fbxw7 is lost to better understand mechanistically the role of Fbxw7 in PDAC progression to metastasis. This study addresses novel facets of PDAC metastasis which has the potential to identify novel therapeutic strategies for advanced and metastatic disease.

Thesis Advisor: Tyler Jacks
Title: David H. Koch Professor of Biology

CURRICULUM VITAE

GRISSEL CERVANTES JARAMILLO

Address: Koch Institute for Integrative Cancer Research
Massachusetts Institute of Technology
77 Massachusetts Ave, 76-453
Cambridge, MA 02139

Email: grisselc@mit.edu

EDUCATION

2017 - 2022 **PhD Candidate**, Harvard-MIT Health Sciences and Technology
Massachusetts Institute of Technology, Cambridge, MA 02139

2020 - 2021 **Healthcare Management Certificate**
MIT Sloan School of Business, Cambridge, MA 02139

2014 - 2017 **B.S. Biochemistry and Molecular Biology**, summa cum laude, departmental honors
University of Miami, Miami, FL 33146

2013 - 2014 Started B.S. Biochemistry and Molecular Biology,
University of Havana, Cuba

RESEARCH / WORK EXPERIENCE

2018 - 2022 **PhD Candidate**
Advisor: Prof. Tyler Jacks
Massachusetts Institute of Technology, Cambridge, MA 02139
Suppression of the ubiquitin ligase function of FBXW7 accelerates metastatic progression of pancreatic cancer.

Discovered that loss of the ubiquitin ligase function of FBXW7 induces metastatic progression in mouse models of pancreatic cancer. Currently investigating the molecular mechanism of action of FBXW7 in PDAC progression to metastasis.

2021 - 2022 **Clinical Rotations**
Mount Auburn Hospital
Harvard Medical School, Cambridge, MA 02138
Studied medical sciences and become fluent in the language and culture of medicine through structured clinical experiences.

2015 - 2017 **Research Assistant / Undergraduate Researcher**
Advisor: Dr. Stephen Lee
University of Miami Miller School of Medicine
Miami, FL 33146

Project 1: Oxygen-sensitive remodeling of central carbon metabolism by eIF-5B.

Identified eIF5B as a hypoxia-specific translation factor involved in translocating met-tRNAiMet to initiating ribosomes, therefore it is involved in translatome reprograming under hypoxic conditions.

Discovered that eIF5B-dependent mRNAs encode proteins of central carbon metabolism, fructolysis, and other pathways enhanced in cells exposed to hypoxic conditions such as solid tumors like glioblastoma.

Project 2 - Symbiosis between ISR and O2-regulated Translatome reprogramming:

Discovered that during hypoxic stress observed in glioblastoma, the Integrated Stress Response (ISR) and the Hypoxic Translatome Reprograming are functionally integrated to control global protein synthesis as an adaptation mechanism of cancer cells to hypoxia.

PROJECTS

2020 - 2021 **Healthcare Lab Project**

MIT Sloan School of Management and Boston Children's Hospital
Cambridge, MA 02139

Determining the Economic Value of Post-Mortem Genetic Testing in Sudden Cardiac Death (SCD) Cases.

Developed a probability adjusted decision tree model to evaluate the financial impact of post-mortem genetic testing after a case of SCD on downstream testing and monitoring costs of family members. Study provides strong support that post-mortem genetic testing after SCD can lead to meaningful cost savings in most family scenarios and insurer types.

2017 - 2022 **Capstone Project in Fundamentals of Drug Development**

MIT Sloan School of Management
Cambridge, MA 02139

Proposed to develop a technology based on cell-penetrating proteins engineered for specificity against a variety of oncogenic proteins called InFUSE, opening a new door for improved cancer therapeutics.

Developed a long-term strategy for the use of the proposed InFUSE platform in our primary indication of interest (Ewing's Sarcoma), from target discovery, to pre-clinical and clinical trials as well as commercialization.

2012 - 2013 University of Havana, Biology Department
Havana, Cuba
Supervisor: Emanuel Mora Macias.
Research Project 1:
Researched topics about Genetically Modified Organisms as a method to control diseases transmitted by mosquitoes. Proposed and developed a research thesis. Acquired knowledge of genetics techniques.
Research Project 2:
Researched topics related with thermoregulation. Collected data of body temperature (iButton) of four women and one man and collected data of menstrual cycle of four women. Analyzed relationship between menstrual cycle and body temperature. Developed abilities of scientific method of research

HONORS AND AWARDS

2019 - 2023 NIH Ruth L. Kirschstein National Research Service Award Individual Predoctoral Fellowship to promote diversity in Health – related research (Parent F31-Diversity) Fellow.

2022 Commitment to a Sustainable Environment Award
Difference Maker Pitch Competition
University of Massachusetts Lowell, Lowell, MA 01854

2021 Dissertation Scholarship
Society of Hispanic Professionals and Engineers

2021 Best Poster Award,
2nd Ubiquitin Function in Health and Disease
Fusion Conference

2021 Mejorando category Winner
SHPE Science Symposium Competition

2020 Graduate Scholarship
Society of Hispanic Professionals and Engineers

2019 Ford Fellowship Honorable Mention

2015 - 2018 Hispanic Scholarship Fund Awardee

PUBLICATIONS

2022 Peter M K Westcott, Francesc Muyas, Haley Hauck, Olivia Smith, Nathan J Sacks, Zackery A Ely, Alex M Jaeger, William M Rideout III, Arjun Bhutkar, Daniel Zhang, Mary C Beytagh, David A Canner, **Grissel C Jaramillo**, Roderick T Bronson, Santiago Naranjo, Abbey Jin, JJ Patten, Amanda M Cruz, Isidro Cortes-Ciriano, Tyler Jacks.

Mismatch repair deficiency is not sufficient to elicit tumor immunogenicity. (2022). Nature (in review)

Zackery A. Ely, Nicolas Mathey-Andrews, Santiago Naranjo, Samuel I. Gould, Christina Cabana, Kim L. Mercer, William M. Rideout III, Gregory A Newby, **Grissel Cervantes Jaramillo**, Katie Holland, Peyton B. Randolph, William A Freed-Pastor, Jessie R. Davis, Peter M.K. Westcott, Andrew V. Anzalone, Francisco J. Sánchez-Rivera, David R. Liu, Tyler Jacks. A prime editor mouse for modeling a broad spectrum of somatic mutations. (2022). Nature Biotechnology (in review)

2021 Hamza B, Miller AB, Meier L, Stockslager M, Ng SR, King EM, Lin L, DeGouveia KL, Mulugeta N, Calistri NL, Strouf H, Bray C, Rodriguez F, Freed-Pastor WA, Chin CR, **Jaramillo GC**, Burger ML, Weinberg RA, Shalek AK, Jacks T, Manalis SR. Measuring kinetics and metastatic propensity of CTCs by blood exchange between mice. Nat Commun. 2021 Sep 28;12(1):5680.

2018 J J David Ho, Nathan C Balukoff, **Grissel Cervantes**, Petrice D Malcolm, Jonathan R Krieger, Stephen Lee(2018) Oxygen-sensitive remodeling of central carbon metabolism by archaic eIF-5B. Cell Reports.

PRESENTATIONS

2022 **Grissel Cervantes**, et. al. Suppression of the Uniquitin Ligase Function of FBXW7 Accelerates Metastatic Progression of Pancreatic Ductal Adenocarcinoma. Jacks Lab 30th Anniversary. **Poster Presentation**.

2021 **Grissel Cervantes**, et. al. Suppression of the Uniquitin Ligase Function of FBXW7 Accelerates Metastatic Progression of Pancreatic Ductal Adenocarcinoma. 2nd Ubiquitin Function in Health and Disease Virtual Conference. **Poster Presentation**.

2020 **Grissel Cervantes**, *et. al.* Suppression of the Uniquitin Ligase Function of FBXW7 Accelerates Metastatic Progression of Pancreatic Ductal Adenocarcinoma. SHPE Science Symposium Competition. **Talk**.

2013 Poster Presentation at the National Academic Forum in Havana, Cuba.

LEADERSHIP AND VOLUNTEER WORK

2021 - 2022	Undergraduate Research Mentor Jacks Lab, Koch Institute for Integrative Cancer Research, Massachusetts Institute of Technology, Cambridge, MA 02139
2020 - 2022	Student Interviewer Harvard - MIT Health Sciences and Technology Program Massachusetts Institute of Technology, Cambridge, MA 02139
2019	Applications Review Committee MIT Summer Research Program (MSRP), Cambridge, MA 02139
2018 - 2022	Tutor, Cambridge School Volunteers Program, Cambridge, MA 02139
2012 - 2013	Teacher of the class “Environmental Management in Cojimar”, Guerrillero Heroico Elemenrary School, Cojimar, Havana, Cuba
2006 - 2008	Committee representative of education and culture Abdala Middle School, Cojimar, Havana, Cuba

ACKNOWLEDGEMENTS

While growing up in Cuba I never imagined that one day I would become a Doctor from MIT and Harvard. I remember watching movies about these schools and it felt unreal and unattainable. Today it is a dream come true and I have many people to thank for their unconditional support, mentorship, and love.

I would like to express my deepest gratitude to my Ph.D. advisor, **Tyler Jacks**, for giving me the incredible opportunity to join the lab and the extended Jacks Lab family that he has fostered for the past 30 years. I came to the lab with little experience in cancer biology, but you still took a chance on me and believed in me, and I will be forever grateful. Your passion and dedication to research and mentees is so inspiring, that it reflects across the Jacks Lab family. You have created an amazing environment for all of us to pursue our interests in cancer research and ask big questions. Your balance of mentorship and independence has helped me grow as a scientist much more than I ever imagined. The way I see this is that you provide us with the strong foundation we need to then choose our own ways to grow, which makes our community so great and unique. I want to thank you for allowing me to explore many different projects and areas. By entrusting me with the freedom and support to do my best, your confidence in me became my own. I also want to thank you for your unconditional support in our career development for our future in and outside the lab. Your dedication to listening to us, our goals, aspirations, and priorities really make each of our interactions very exciting. I'm honored and privileged to have been a part of this community and to have learned from you during this time how to be a great leader. I hope I made (and continue to make) you proud!!

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I want to thank all the members of the Jacks Lab, past and current. You have all played an important part in my journey and I will be forever grateful. To **Will Freed-Pastor**, thank you so much for your mentorship and countless hours showing me the world of pancreatic cancer research. To the first group of graduate students that I had the privilege of working alongside **Santiago, Rodrigo, Sheng Rong, Laurens, Amy, David, Amanda,** and **Ryan**, thank you so much for creating such a welcoming environment and for always supporting me and having my back. I'm honored to have you all as friends. To the best baymates I could ask for: **Carla, Megan, and Laurens**, thank you for always being there to listen in the good and bad times, always with great words of advice both scientifically and personally. To **Peter, Lindsay, Caterina, Demi, Britt, Will Hwang, Hannah, Jennifer, Emma Sanders, Zach Kulstad, Anna** and **Nimisha** thank you so much for everything, you have all helped me in my journey though graduate school in many ways. To **Lin Lin**, thank you so much for being the most amazing partner for surgeries, your light shines so bright that it makes every long day of work very

enjoyable. To the current group of graduate students who I'm also lucky to call as friends: **Emma Rose, Christina, Sean-Luc**, **Nick,** and **Zack**, thank you for being amazing and for your unconditional support and love! It has been a blessing watching you grow in the lab and I can't wait to see all the amazing things you will continue to do inside and outside of the lab. I couldn't ask for a better group of grad students in crime to close my Ph.D. work. We have been together through a big transition in the lab, and I know that the lab is in great hands with all of you! To my mentee **Javier Villa Ortiz,** thank you so much for all your contributions to the project, but also for teaching me so much from how to be a better mentor to looking at my project with an engineer's point of view. I'm proud of all your accomplishments and I can't wait to see what the future holds for you.

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To collaborators, thank you **AJ** and **Sarah Blatt** for your patience teaching me data science. To **Alex Miller** and **Bashar Hamza**, thank you for being such amazing people and collaborators. To **Kathy Cormier**, thank you for your devoted help at the histology core, even if that means a phone call at night. To **Nicole Henning**, thank you so much for your passion and enthusiasm, and to **Richard Schiavoni** thank you so much for all the help with the proteomics data.

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To HST's administrative staff: **Laurie** and **Julie**, and to HST's admissions committee, thank you so much for seeing something in me and for always being there. Thank you for giving this incredible opportunity to this girl from a small town that no one knew much about. I will be forever grateful and I hope I made you proud! To my HST friends: **Claudia, Shoshana, Ashwin, Lily, Emily, Rebecca, Kathy, Indie, Brenan,** and **Taylor**, I couldn't ask for a better cohort of friends to go through grad school together, you are all an inspiration and I can't wait to see all the amazing things you will do next.

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To my dear friends from Cuba **Jessica, Giselle, Deborah, and Marianela**. Having recently moved from Cuba to different places in the world and seeing you grow has been one of the most inspiring things I have experienced. I'm incredibly proud of you, and I hope that I've made you proud as well. Thank you so much for your continued support, even when I disappear from the phone for weeks. You have always been there for me in good and bad times, always supporting and giving me great advice. We have been together through many things since we were kids and I consider you my family. I can't wait to grow old together and

see all the amazing things you will continue to accomplish, las adoro!!! To el **Tony, Jeffrey, Ceidis, Leo,** and **Aimelis**, thank you for your unconditional love. To **Tania, Brenda,** and **Ashley**, my honorary lab friends, thank you so much for being such amazing friends.

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kindness, and generosity. I miss all of you immensely, and I hope you are watching down so that I can make you proud.

Words cannot express my gratitude for my parents, Raul and Moraima, mi **Papito y Mamita**. You are an inspiration to me and my role models. Seeing how much you have accomplished in your lives coming from a place with almost nothing, is my biggest motivation. Thank you for being so caring and loving, with me and everyone around us. Thank you for your humility and dedication, you have always taught me by example. Thank you for always pushing me to pursue my dreams and interests. You taught me that with passion, humility, hard work, and dedication I could achieve anything I set my mind to. Thank you for always believing in me, your confidence in me became mine. Thank you for teaching me to always shoot for the stars and never give up. I would not be here at Harvard and MIT without your support. You have been my rock throughout this journey. Thank you for risking everything coming to the US to give me a better future. You gave me all the support I needed to focus on my career, to grow professionally and personally. You gave me the freedom to explore different paths in my career and always pushed me to follow my passion. Thank you for always pushing me beyond my limits to be the better version of myself. I've grown into the person I'm today thanks to you. Thank you for teaching me the importance of family and showing me to have fun and enjoy life. Thank you for teaching me to be resourceful and independent and to value myself. I hope that I have made (and continue to make) you proud. Love you with all my heart!

To my **husband Ariel**, my partner in crime, my bebecito, my jevi, my titi, my amuchi, my puchi, and my nenito. Thank you for being a constant source of support and encouragement since high school and all the way through the challenges of graduate school and life. Thank you for all the things you do for me, from bringing dinner to my late nights at work, helping outline tumors in countless images, taking notes in the mouse-scented lab as I work to speed up my work, to just seating next to me to keep me company in many late nights. Thank you so much for teaching me data analysis and command lines for the first time ever. You have been so patient with me when I'm frustrated, you celebrate with me when the little things go right, and you are there whenever I need you to just listen. Thank you for always reminding me to stay true to myself and believe in myself. Your drive in life is such an inspiration to me. Coming from nothing in Cuba, you decided to take on the most dangerous journey by yourself to get here to the US. Fast forward 6 years and you have accomplished so much, dual degree, master's degree, and a dream job, but the funny thing is, you are far from done, and you motivate me to do the same and continue growing. I can't wait to grow old together and have a beautiful family with you!!! Love you to the moon and back! We started with our beautiful fur baby, **Dante**, the craziest but most loving Labrador we will ever meet. Dante, I can't thank you enough for all of the joy you have brought into our lives these past 5 years. Our time together has been filled with silly memories like eating everything, but also lots and lots of kisses and cuddles. Thank you for always putting a smile on us, no matter how good or bad a day we are having. Thank you for being so active, running around the house to retrieve a napkin or a shoe counted towards our daily dose of cardio and calorie count. Thank you for teaching us perspective. Too often we are very stressed, but every time we are with you, you remind us to enjoy the day, breathe and be happy. To our new fur baby **Luna**, only two weeks with us you have already brought so much joy to our lives. Thank you both for being our best friends. We love you so much our giant tribis!!!

This thesis is dedicated to my amazing parents, Raul and Moraima; my loving husband, Ariel; and my beautiful fur baby, Dante. You all deserve this Ph.D. Love you!

TABLE OF CONTENTS

CHAPTER 1

INTRODUCTION

PART 1: Pancreatic Cancer

1.1 Introduction to Pancreatic Cancer

The first recorded description of pancreatic cancer is accredited to Giovanni Battista Morgagni's work in 1761, titled 'de Sedibus Et Causis Morborum Per Anatomen Indagatis Libri Quinque'. However, the accuracy of the diagnosis of adenocarcinoma is questionable due to the absence of microscopic examination. The next significant advance in our understanding of pancreatic cancer came in 1858, when Jacob Mendez Da Costa reviewed Morgagni's initial study and provided the first microscopic diagnosis of adenocarcinoma, establishing pancreatic cancer as a distinct disease entity (Da Costa, 1858).

The exocrine and endocrine functions of the pancreas make it a very complex organ. The endocrine pancreas consists of hormone-secreting endocrine islets and relatively inactive stellate cells (Figure 1). Diabetes mellitus develops when the endocrine pancreas fails to secrete the hormone insulin (Kleeff et al., 2016; Ellis et al., 2017). On the other hand, the exocrine pancreas is made up of acinar cells that produce digesting enzymes, ductal cells that release bicarbonate, and centroacinar cells that exist in the transition between acinar and ductal cells (Figure 1). Exocrine function is impaired in several disorders, including chronic pancreatitis, cystic fibrosis, and cancer (Kleef et al., 2016; Ellis et al., 2017). All of these pathologies result in devastating personal and economic consequences, in particular pancreatic cancer.

Pancreatic cancer is the most lethal common malignancy because it is usually diagnosed at an advanced stage and is frequently treatment-resistant. More than 90%

of pancreatic neoplasms are pancreatic ductal adenocarcinomas (PDAC). Other rare pancreas malignancies include neuroendocrine tumors and acinar carcinomas, and even less prevalent are colloid carcinomas, pancreatoblastomas, and solid-pseudo papillary neoplasms (Ryan et al., 2014; Kleeff et al., 2016).

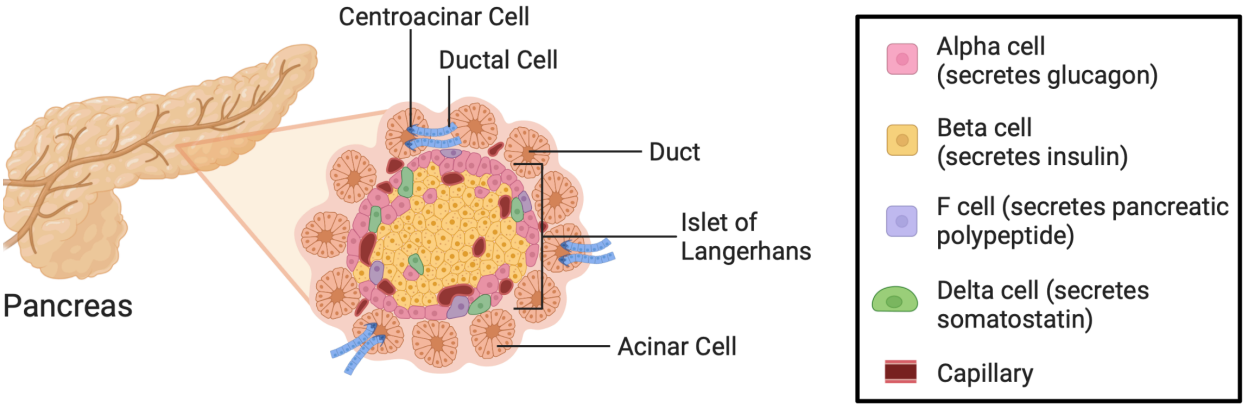


Figure 1: **Major cell types and structures of the pancreas**. The pancreas is composed of exocrine components (acinar cells, ductal cells, and centroacinar cells) and endocrine components (islet of Langerhans composed of alpha cells, beta cells, F cells, and delta cells). Figure 8dapted from Ellis *et al.*, 2017. Modified from "Pancreatic Islet of Langerhans" template, by BioRender.com (2022). Retrieved from https://app.biorender.com/biorender-templates.

1.1.1 Epidemiology and Risk Factors

The global incidence of pancreatic cancer ranges from 1 to 10 cases per 100,000 individuals, with an anticipated 495,773 people diagnosed with the disease in 2020. (Cancer Facts & Figures 2022; SEET Cancer Stats Facts). This year, an estimated 62,210 adults in the United States will be diagnosed with pancreatic cancer, accounting for 3% of all malignancies (American Cancer Society, SEER Cancer Stats Facts). Pancreatic cancer is the eighth most prevalent cancer among women and the tenth most common cancer among men. In addition, it is estimated that 49,830 deaths from this disease will occur in the United States this year, making pancreatic cancer the

third leading cause of cancer deaths and it is predicted to rise to the second leading cause of cancer-related deaths in the coming ten years (American Cancer Society, SEER Cancer Stats Facts).

A quarter to a third of pancreatic cancer cases are attributable to risk factors like chronic pancreatitis, type 2 diabetes, and tobacco use (Kleef *et al.*, 2016; Ryan *et al.*, 2014; Klein *et al.*, 2004). Smokers have from twice to three times higher risk of presenting with pancreatic cancer in their lifetime than non-smokers, accounting for approximately 15-30% of cases in several populations (Kleef *et al.*, 2016). Obesity, low physical activity, and diet like excessive saturated fats, low fruits and vegetables, and incorporation of red and processed meats are also linked to a higher risk of developing pancreatic cancer (Genkinger *et al.*, 2015; Kleef *et al.*, 2016). In addition, heavy alcohol consumption has been associated with an increased risk by causing chronic pancreatitis, leading to a tenfold increase in the risk of developing pancreatic cancer (Genkinger *et al.*, 2015). Furthermore, about 5-10% of pancreatic cancers have an inherited component, however, the genetic basis of familial cases has not been identified in many patients (Ryan *et al.*, 2014; Klein *et al.*, 2004). A known family history of pancreatic cancer in a first-degree relative increases the risk of developing pancreatic cancer, compared to the general population, by a factor of up to 30 depending on how many relatives are affected. Therefore, frequent screening of patients with an inherited predisposition is valuable for early diagnoses and treatment. However, these screening techniques have shown no value for asymptomatic patients.

The 5-year survival rate for these patients remains poor at 11.5% despite scientific advancements in the understanding of PDAC tumor biology and the

development of innovative therapy regimens. (SEER Cancer Stats Facts). Cancer stage at diagnosis determines treatment options and has a strong influence on the length of survival. In general, the earlier pancreatic cancer is caught, the better chance the person has of surviving 5-year. If a patient presents to the clinic with localized disease, where the primary tumor is confined to the pancreas (12% of patients), the 5-year survival rate is 43.9%. This drastically worsens if the patient presents with regional disease, where the primary tumor has spread to the regional lymph nodes (30% of patients) and the 5-year survival rate decreases to 14.7%. Unfortunately, most patients (52%) present to the clinic with advanced and metastatic disease, for which the 5-year survival rate is only 3.1% (SEER Cancer Stats Facts).

1.1.2 Clinical Presentation, Diagnosis, and Staging

The presenting signs and symptoms of patients with pancreatic cancer change based on the location of the tumor. About 60-70% of PDAC tumors arise in the head of the pancreas, and only 20-25% are located in the body and tail of the pancreas (Ryan *et al.*, 2014). These lesions can vary in size from microscopic lesions to masses over 10cm (Sanchez *et al.*, 2015). While tumors of the tail and body of the pancreas often obstruct distal portions of the pancreatic ducts, tumors of the head of the pancreas typically block both the pancreatic ducts and the bile ducts, causing upstream dilatation (Kleeff *et al.*, 2016). Patients with PDAC most commonly present with abdominal pain, weight loss, asthenia, and anorexia (Ryan *et al.*, 2014; Chari *et al.*, 2005; Porta *et al.*, 2005). Those patients with tumors in the head of the pancreas most

commonly present with jaundice (Porta *et al.*, 2005). In addition, about 50% of patients with PDAC present with diabetes.

When a mass is detected, abdominal computer tomography with both arterial and venous phases are usually sufficient to determine the initial stage and treatment for the patient (Ryan *et al.*, 2014). Pancreatic phase images assess pancreas lesions, arterial phase images assess coeliac trunk, superior mesenteric artery, and other artery involvement, and portal venous phase images assess portal vein, superior mesenteric vein, and other vein involvement. (Kleeff *et al.*, 2016). However, pathology is required to establish a definitive diagnosis, therefore, a biopsy of the primary tumor is often done using endoscopic ultrasonography and fine-needle aspiration (Ryan *et al.*, 2014; Kleeff *et al.*, 2016). The earliest stage of pancreas cancer is Stage 1 or carcinoma in situ where the tumor is confined within the pancreas. Then, stages range from 2 to 4 as the tumors become more invasive (Figure 2) (American Cancer Society).

Unfortunately, there are no sensitive or specific biomarkers up to date for the screening and diagnosis of PDAC. Some tumor markers, such as carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9), if elevated, are useful in following patients with known disease (Ryan *et al.*, 2014). Similarly, the presence of circulating tumor cells could be diagnostic, however, these are only present in only some patients with metastatic disease. Interestingly, Sausen *et. al.* showed that circulating tumor DNA encoding mutant KRAS has been detected in 43% of patients with localized disease at the time of diagnosis, demonstrating the power of testing circulating tumor DNA with a panel of mutated genes relevant to the disease for better diagnosis. In addition, potential biomarkers for early pancreatic cancer diagnosis have

been found in various studies. For instance, Mirus et al. used sequential plasma samples from pre-diagnosis patients and from genetically engineered mouse models (GEMMs) with pancreatic tumors and performed antibody microarrays, which produced a highly specific signature made up of tenascin C and the estrogen receptor 1 (HER1). In addition, Melo et al. found that both GEMMs and patients with early-stage disease have the heparin surface proteoglycan glypican 1 on the outer layer of circulating exosomes. These exosomes also contain other proteins, nucleic acids, and microRNAs that could yield additional diagnostic benefits. For instance, in those patients that developed type 3c diabetes mellitus years before being diagnosed with pancreatic cancer, the exosomes contained adrenomedullin (Javeed *et al.*, 2015) Therefore, measuring exosome-bound adrenomedullin in people with type 3c diabetes may aid in the earlier detection of pancreatic cancer. Lastly, studies have shown that plasma microRNA signatures have diagnostic potential for pancreatic cancer, and therefore, such assays should be improved to enable early detection (Johnston *et al.*, 2009; Zhang *et al.*, 2013).

Sadly, more than 90% of patients die from the disease after being diagnosed. About 70% of these individuals pass away from extensive metastatic disease, while the remaining 30% pass away from modest metastatic disease but typically have significant primary tumors. Therefore, there is a significant need to better understand the biology behind PDAC progression to metastasis for better treatment options for these patients.

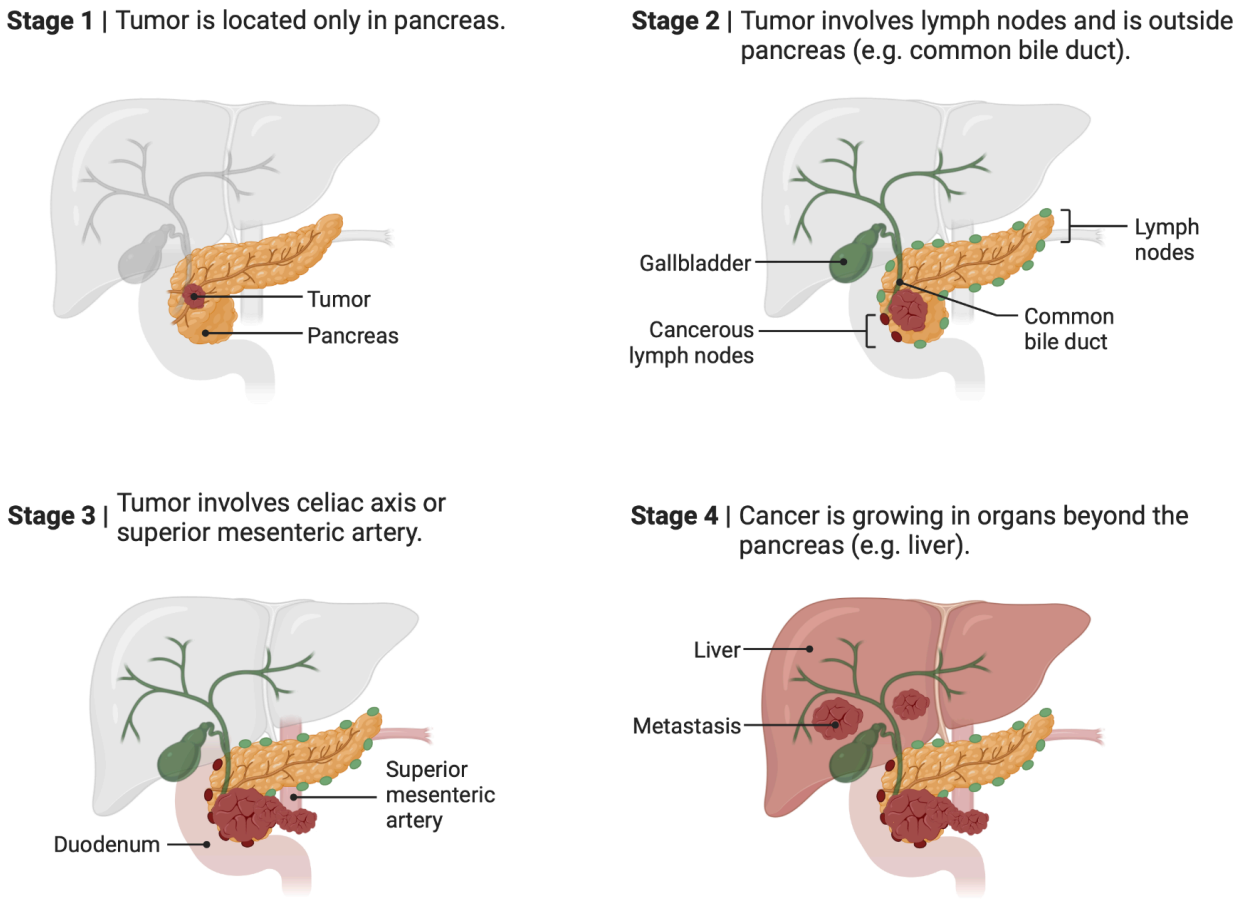


Figure 2: Pancreatic cancer staging. Stages are defined based on the invasiveness of the tumor at the time of diagnosis. A description of the different stages can be found in the figure. Reprinted from "Pancreatic cancer staging" template, by BioRender.com (2022). Retrieved from https://app.biorender.com/biorender-templates.

1.1.3 Disease Management

Surgical resection in combination with chemotherapy is the current best treatment option for pancreatic cancer patients. The advantages of surgical removal of a PDAC tumor were questioned a few decades ago due to unacceptably high morbidity and mortality rates, especially at small medical centers (Bramhall *et al.*, 1995; Tjarda Van Heek *et al.*, 2005). This led to skepticism and underutilization of PDAC tumor surgical resection. Nowadays, Pancreatic cancer surgery can now be performed safely

with a 5% mortality rate thanks to technological advancements, rigorous training, increasing experience, further centralization of these cases to high-volume centers, and improved preoperative management. As a result, surgical resection is the only potentially curative option; however, only patients whose cancer cells have not migrated to important abdominal arteries and nearby organs may benefit from this. For this reason, the treatment regime is determined by disease stage:

Stages 1 and 2 - Surgically resectable PDAC:

PDAC stages 1 and 2 are tumors restricted to the pancreas, regardless of the involvement of the draining lymph nodes. The involvement of local arteries in these tumors determines where patients fall on a continuum from resectable to unresectable tumors. In order to determine respectability, it is crucial to evaluate the underlying tumor and the involvement of the local vessels, such as the celiac artery, superior mesenteric artery and vein, portal vein, and hepatic artery. Only 10 to 20 percent of patients have resectable illness at the time of diagnosis, making them candidates for surgery (Ryan et al., 2014; Kleeff et al., 2016). A partial pancreaticoduodenectomy, often known as the Whipple procedure, is typically necessary for tumors near the head of the pancreas, with or without a partial resection of the distal stomach. Distal pancreatectomy with splenectomy is performed for pancreatic tail tumors (Ryan et al., 2014; Kleeff et al., 2016). Except for malignancies spanning the entire length of the organ or for tumors positioned in the center, a total pancreatectomy is rarely necessary since it is accompanied by exocrine and endocrine insufficiency (brittle diabetes mellitus).

Unfortunately, after resection, most of these patients have microscopically positive margins and studies have shown that surgery alone is associated with poor long-term outcomes. Therefore, surgery is accompanied by adjuvant therapy. Adjuvant therapy includes systemic therapy to reduce distant metastases and chemo-radiotherapy to reduce the risk of locoregional failure (Ryan *et al.*, 2014). Studies have established a 6 months treatment regime with either gemcitabine or fluorouracil significantly improving overall survival, however, the use of radiation therapy is controversial in the field.

Stage 3 - Locally advanced, unresectable PDAC:

For patients diagnosed with borderline resectable and locally advanced disease, neoadjuvant therapy is the first line of treatment that could be followed up by surgical resection. About 30-40% of patients present with borderline resectable and locally advanced disease. In the past years, there has been growing interest in incorporating multi-agent chemotherapy regimes for these patients, such as fluoracil, irinotecan, oxaliplatin and leucovorin (FOLFIRINOX), and gemcitabine plus albumin-bound paclitaxel particles (nab-paclitaxel) in preoperative and postoperative regimes for patients with more advanced disease (Ryan *et al.*, 2014; Kleeff *et al.*, 2016). Some of these patients regress to localized disease and surgical resection becomes an option.

Stage 4 - Metastatic PDAC:

The majority of patients present to the clinic at this advanced and metastatic stage with many symptoms, and as such, supportive care is critical in helping these

patients remain well for as long as possible. Fluorouracil-based chemotherapies, especially FOLFIRINIX improves the survival of these patients. Survival for 2 years was previously rare among patients with metastatic disease and now it is seen in approximately 10% of these patients when treated with FOLFIRINOX or gemcitabine-nab-paclitaxel (Ryan *et al.*, 2014). Unfortunately, these patients rapidly succumb to tumor spread and vital organ dysfunction, intractable pain, galloping cachexia, and coagulopathy.

There is now significant potential for new therapies for metastatic pancreatic cancer.. These include RAS-directed therapies, immunotherapies, and stroma-modifying therapies. Novel $KRAS^{G12C}$ inhibitors are now becoming available for patients with KRASG12C mutant tumors. Even though altered forms of the KRAS gene are found in more than 90% of pancreatic cancers, only a few pancreatic cancer patients have this particular mutation. However, this inhibitor shows great promise for this subset of patients, and researchers are actively looking for other forms of targeted therapies. Immunotherapies also hold great promise in the treatment of PDAC. An immune checkpoint inhibitor like pembrolizumab, for instance, has been licensed for use in patients with pancreatic cancer with substantial microsatellite instability. Combining immunotherapies has significant promise for the treatment of PDAC. For instance, patients with advanced pancreatic cancer may benefit from taking immune checkpoint inhibitors along with a CD40 agonist, a type of medication that helps activate T cells. Last but not least, stroma in pancreatic malignancies is substantially denser than in most tumors. More chemotherapy medicines may reach cancer cells if substances that aid in stroma breakdown are used.

1.2 Pathophysiology

Pancreatic cancer follows a step-wise progression caused by the gradual accumulation of genetic mutations. Due to activating mutations of oncogenic KRAS (occurring in more than 90% of cases), a normal pancreatic duct transforms into a pre-invasive precursor lesion known as pancreatic intraepithelial neoplasia (PanIN). The cytoplasm of the normal ductal and ductular epithelium is cuboidal to low-columnar in shape, and lacks atypic, nuclear crowding, and mucinous cytoplasm (Olca et al., 2015; Basturk et al., 2001). Low-grade PanIN is divided into three categories as they advance: PanIN-1A, PanIN-1B, PanIN-2, and PanIN-3 (Figure 3). The hallmarks of PanIN-1A is the presence of flat epithelial lesions made up of tall columnar cells with basally positioned nuclei and an abundance of supra nuclear mucin (Sanchez *et al.*, 2015). PanIN-1B is the same as PanIN-1A except that it possesses papillary, micropapillary, or basally pseudo stratified architecture (Sanchez *et al.*, 2015). Mutinous epithelial lesions known as PanIN-2 can be papillary or flat, and their nuclei may exhibit abnormalities such loss of polarity, nuclear crowding, enlargement, pseudo-stratification, and hyperchromatism (Sanchez et al., 2015). PanIN-3, which also exhibits real cribriforming luminal necrosis and significant cytologic abnormalities, magnifies these nuclear abnormalities further. Luminal necrosis and papillary morphology are typical features of PanIN-3, which typically form papillary or micropapillary tiny clusters of epithelial cells. These lesions exhibit aberrant mitosis, which can infrequently occur, loss of nuclear polarity, dystrophic giblet cells (goblet cells with nuclei orientated towards the lumen and mutinous cytoplasm oriented towards the basement membrane), large (macro) nucleoli, and nuclear abnormalities (Sanchez et al., 2015).

These pre-invasive lesions can ultimately develop into an invasive PDAC due to the loss of function of tumor suppressor genes such as TP53, CDKN2A, and SMAD4 which can then invade and metastasize to distant sites (Figure 3). Studies have not yet identified a recurrent metastasis-specific mutation, so there is still a need to better understand PDAC progression to metastasis for better treatment options for these patients. PDAC typically metastasizes to regional lymph nodes, then to the liver, and less commonly to the lungs, kidneys, and adrenal glands. PDAC is made up of atypical glands that resemble medium-sized or smaller pancreatic ducts (Haeberle *et al.*, 2019). However, the differences in growth patterns between and within tumors are startling. PDAC may contain non-tubular elements such clear-cell, cribriform, or gyriform elements, which could affect a patient's prognosis (Sanchez et al., 2015; Haeberle et al., 2019). This neoplasm's aggressive biological nature is influenced by the fact that these lesions are frequently poorly differentiated and immersed in a highly desmoplastic stroma made up of stromal cells, inflammatory cells, and extracellular matrix proteins.

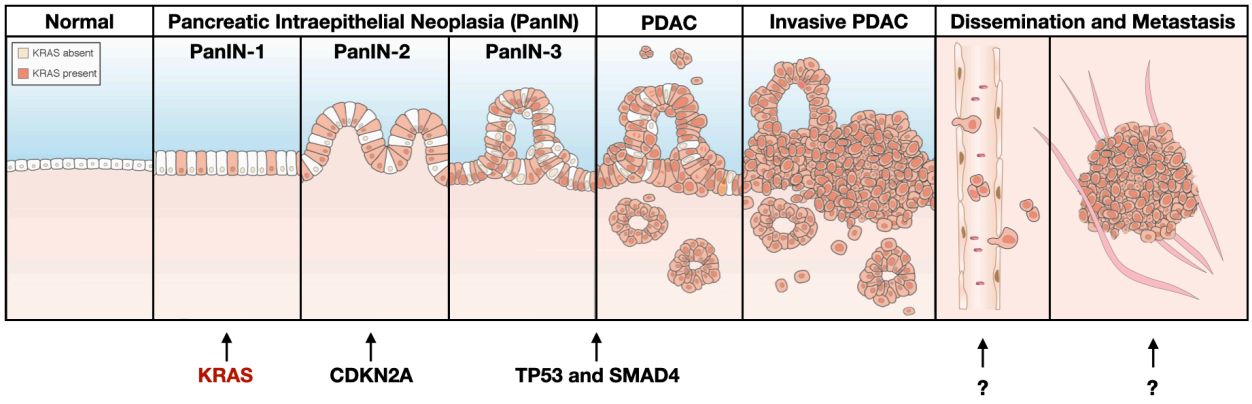


Figure 3: Genetic Progression of PDAC. PDAC starts with the transition of normal cells to pre-invasive lesions known as Pancreatic Intraepithelial Neoplasia (PanIN) due to activating mutations of oncogenic Kras. These lesions progress to invasive PDAC after the loss of tumor suppressors such as TP53, CDKN2A, and SMAD4.

1.2.1 Aberrant Signaling Pathways in Pancreatic Cancer

In pancreatic cancer, there are several abnormal nodes and crosstalk between pathways that complicate the signaling process. PDAC shows abnormal autocrine and paracrine signaling cascades that promote signaling molecules like transforming growth factor (TGF), insulin-like growth factor 1 (IGF1), fibroblast growth factors (FGFs), and hepatocyte growth factor (HGF), as well as their corresponding tyrosine kinase receptors like epidermal growth factor receptor (EGFR), receptor tyrosine protein kinase erbB-2 (ERBB2) (Preis et al., 2011; Kleeff et al., 2016). Along with these pathways, anti-apoptotic and pro-survival pathways such as signal transducer and activator of transcription 3 (STAT3) and nuclear factor-κB (NF-κB) are co-activated. Some pancreatic tumors also reactivate genes including WNT, SHH, and NOTCH that are normally active throughout development (Kleeff et al., 2016; Magliano et al., 2007).

In the context of oncogenic KRAS and CDKN2A loss, PDAC frequently shows increased activity of HGFR and EGFR, increased expression of neurophilin 1, CD44, and integrin 1, which worsen by the gained HGFR ability to form heterodimers with EGFR (Kleeff *et al.*, 2016). Additionally, metabolic changes and resistance to growth-inhibitory pathways are present in pancreatic cancer. The most prominent example of this is aberrant TGF-β signaling as a result from increased production of TGF-β isoforms (Gore et al., 2014). TGF-β is known to be a physiological tumor suppressor, however, it accelerates the growth and metastasis f pancreatic cancer and many other solid tumors through its effects on the tumor microenvironment. TGF-β may also increase the expression of WNT7B through traditional SMAD4-dependent processes, as well as by activating non-canonical signaling through the activation of proto-

oncogene tyrosine-protein kinase Src (SRC), mitogen-activated protein kinase (MAPK), and AKT (Gore et al., 2014; Kleeff et al., 2016).

1.2.2 PDAC Genetics

As was already indicated, activating mutations in KRAS, which are present in more than 90% of tumors, dominate the molecular pathogenesis of PDAC (Figure 4). The KRAS protein is a small GTPase that interacts with growth factor receptors on cell membranes and regulates the switch of numerous signaling pathways and physiological functions. In PDAC, the most frequent KRAS mutations is seen in G12 of exon 2, G12D present in 40% of patients and G12C in 33% of patients (Hu et al., 2021). According to multiple studies, oncogenic KRAS mutations drive PDAC initiation and progression through the different PanIN stages, with mutational frequency from 50% in PanINs to 95% in PDAC (Pasca di Magliano and Logsdon, 2013; Ryan *et al.,* 2014; Hu *et al.*, 2021). However, a major question that remains in the field is how KRAS mutations lead to the initiation and development of pancreatic tumors since studies have shown that KRAS mutations alone are sufficient to induce PanIN lesions in PDAC mouse models (Not *et al.*, 2017; Hingorani *et al.*, 2003). According to studies, elements in the tumor microenvironment such TGF-β, oxidants, and inflammation that promote and activate KRAS also help this transformation process.

Approximately 50-80% of PDAC patients have inactivating mutations in TP53, CDKN2A and SMAD4, while other genes such as MUC16 and ARID1A are mutated in less than 10% of tumors (Figure 4) (TCGA).

Cyclin-dependent kinase inhibitor 2A (CDKN2A) genetic alterations are present in 30-50% of PDAC patients, which collaborates with oncogenic KRAS in driving the malignant transformation (Hu *et al.*, 2021). In pancreatic cancer, deletions, mutations, and promoter hypermethylation are the most common genetic changes in CDKN2A. (Caldas et al., 1994). By directly or indirectly targeting CDK4/6-cyclins, CDKN2A plays a critical part in regulating the cell cycle. Both the p14ARF and p16INK4A proteins are encoded by CDKN2A; they share exons 2 and 3, but differ in exon 1, resulting in two proteins that work in different ways. While p16INK4A induces cell cycle arrest by connecting to and inactivating MDM2, an E3 ubiquitin ligase that mediates p53 deviation, p14ARF binds to CDK4/6 and suppresses the activation of D-cyclins, restricting cell cycle entry (Kong et al., 2016; Kim and Sharpless, 2006).

The most frequently mutated tumor suppressor gene across all malignancies is TP53, and in the case of pancreatic cancer, it is found in 60-70% of patients (Hu *et al.*, 2021). The p53 protein binds to specific DNA sequences and regulates the transcription of downstream genes involved in a myriad of cellular processes such as cell cycle, mitochondrial respiration, cell metabolism, autophagy, and stem cell maintenance and development (Junttila and Evan, 2009; Mantovani *et al.*, 2018). Consistent with its function, TP53 is frequently mutated in its DNA binding domain, where the majority of the mutations are missense, giving cancer cells a great chance to proliferate and survive the hostile environment of the tumor. p53 genetic alterations without loss of heterozygozity have been detected in early PanIN, and homozygous p53 mutations have been observed in PanIN-3, indicating that p53 has the potential to drive PDAC carcinogenesis (Morton et al., 2009; Hu et al., 2021). In fact, Morton *et al.*

constructed a mouse model with mutant-p53 ($Trp53^{R172H}$) in a $Kras^{G12D}$ background and found that $Trp53^{R172H}$ facilitated the transition from premalignant lesions to metastatic PDAC, accompanied by high chromosomal instability (Hingorani *et al.,* 2005).

Lastly, the tumor suppressor SMAD4 (Sma (Caenorhabditis elegans) mothers against decapentaplegia homologue 4) is commonly altered in a myriad of diseases and cancer types, particularly pancreatic cancer, which has a mutation rate of 20–50%. (Hu et al., 2021). Though SMAD4 is not necessary for TGF-β activation, it is essential for eliciting a potent signaling response, since the SMAD family plays a vital function in modulating TGF-β signaling (Warn, 2009). The TGF-β/SMAD4 pathway mediates the proliferation of cancer cells by promoting cell cycle arrest, apoptosis, and DNA damage repair, therefore, genetic alterations in SMAD4 reduce the tumor suppressor activity of the TGF-β pathway (Warn, 2009; Zhao et al., 2018). In addition, it is thought that the epithelial-to-mesenchymal transition (EMT) process in a SMAD4-dependent manner promotes cancer cell invasion and metastasis (Held et al., 2012). Studies have shown that 30% of PDAC patients had homozygous deletions, and 20% have chromosomal allelic loss of SMAD4 (Hu et al., 2021). High-grade precursor lesions have been found to lack SMAD4, indicating that the inactivation of SMAD4 encourages progression to a later stage of carcinogenesis (Wilentz et al., 2000).

On the other hand, among the thousands of infrequently mutated genes, often found at a prevalence of less than 2%, only few genes stand out. Therefore, it is crucial to do research into how these genes are involved in pancreatic cancer in order to develop new clinical practices.

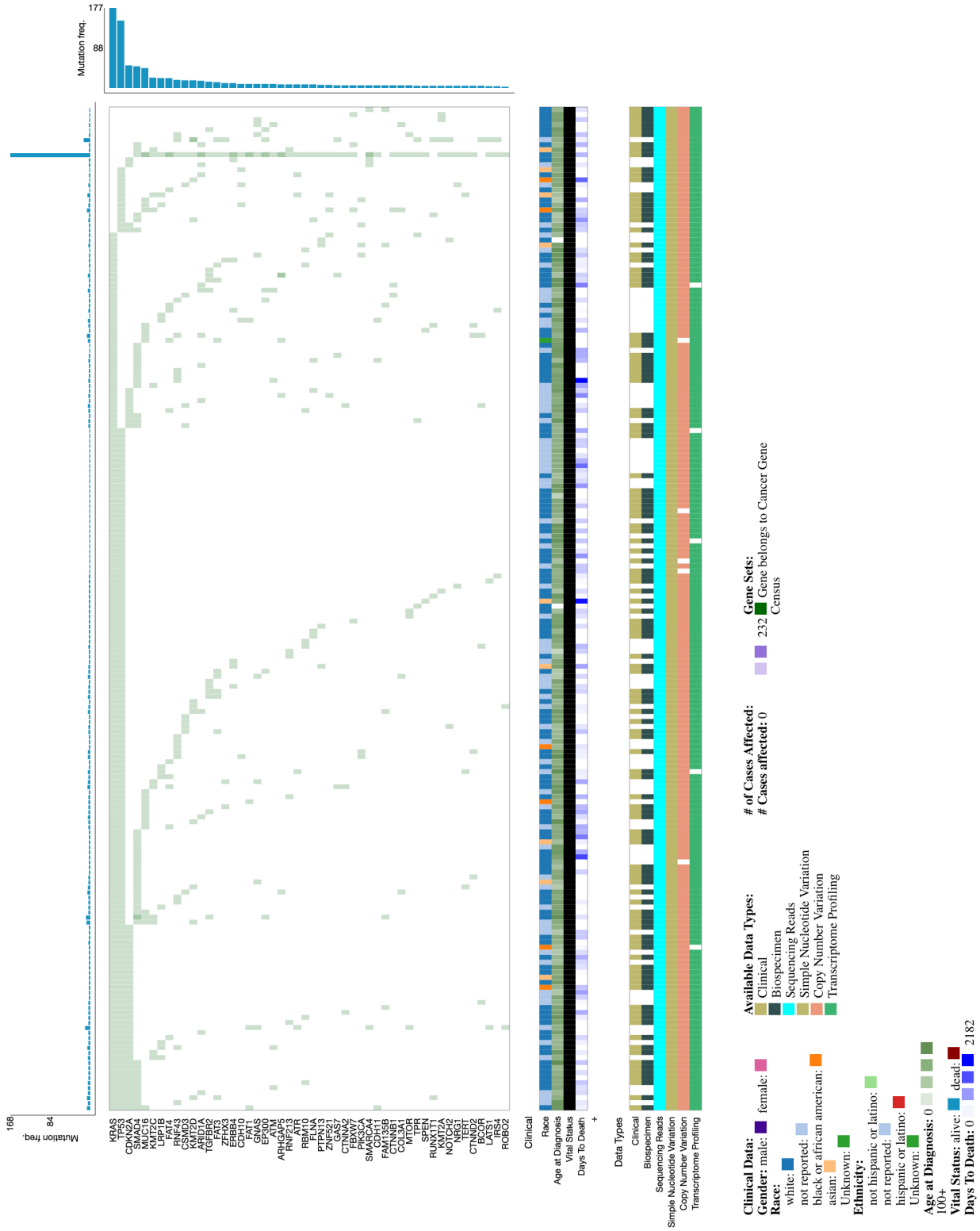


Figure 4: Mutational Landscape of Pancreatic Cancer. 200 most mutated cases and top 50 mutated genes in pancreatic cancer. TCGA Genome Data Analysis Center.

1.3 PDAC Models

Our understanding of pancreatic cancer has considerably increased thanks to the accessibility of model systems for this disease. Preclinical testing has traditionally been conducted mostly in vitro, using two-dimensional cell culture assays, or in vivo, using xenografts or animal models such as genetically engineered mouse models (GEMMs). A more contemporary hybrid approach, known as organoids, has recently taken force where progenitor cells are cultivated in three dimensions, combining both in vitro culture's controllability and simplicity with the potential to recreate niches more similar to PDAC microenvironment.

1.3.1 Genetically Engineered Mouse Models

Transgenic models facilitate the ectopic and temporal expression of specific genes in the mouse genome. The use of tissue or cell-type specific promoters allows great specificity to these systems. In the case of PDAC, lineage-specific promoters include the pancreatic and duodenal homeobox 1 (Pdx1), neuron (Ngn3), and elastase (Ela), among others, which have all been used in GEMMS of PDAC (Miquel et al., 2021). (Supplementary Table 1). These authoctonous models faithfully reproduce the pathophysiology of human illness and have been used to show the roles of numerous mutant genes previously discovered in the human pancreatic cancer genome.

The first GEMMs of PDAC, known as KC mice (Hingorani *et al.*, 2005), presented with the conditional expression of oncogenic $Kras^{G12D}$ in epithelial cells of pancreatic lineage. They demonstrated that oncogenic KRAS alone is sufficient to initiate PanIN lesions, which then spontaneuously develop locally invasive and metastatic pancreatic

cancer. When oncogenic KRAS is combined with additional orthologous mutations in the canonical tumor suppressors CDKN2A, TP53, or SMAD4, pancreatic cancer progression is drastically accelerated, reconstituting the whole spectrum of tumor progression (Aguirre *et al.,* 2003; Hingorani *et al.,* 2005; Izeradjene *et al.,* 2007). These strategies are standard methods to query the function of a potentially pathogenetic allele, however, this is a very labor-intensive method to interrogate novel targets.

PDX1-Cre, LSL-Kras$^{G12D/+}$ and P48$^{+/Cre}$, LSL-Kras$^{G12D/+}$ (KC) transgenic model

In numerous GEMMs, the PDX-1 and p48 promoters have been utilized to study pancreatic cancer. The first identifiable progenitor cell of the pancreas appears in the dorsal and ventral endoderm during murine embryonic day 8 where PDX-1 begins to be expressed (around E8.5). On the other hand, P48 is expressed later in development and is needed to commit cells to a pancreatic cell fate (Offield et al., 1996; Kawaguchi et al., 2002).

Hingorani et al. (2005) KC mouse model expresses a Cre-activated Kras^{G12D} allele in the endogenous Kras locus, LoxP-STOP-LoxP KRAS$^{G12D/+}$ (LSL-KRAS$^{G12D/+}$), and a Cre-recombinase allele driven by pancreas specific promoters PDX-1 or Ptf1-p48. These KC animals developed ductal lesions that recapitulate all three stages of human PanINs. Early PanIN-1A lesions can be seen in mice as young as two weeks old, and higher-grade PanINs are more frequently seen as the mice get older. In many older mice, the pancreas has extensive ductal lesions, and the acinar cells have been replaced by stromal, desmoplastic fibroblasts and inflammatory cells. Finally, these

mice develop invasive and metastatic ductal adenocarcinoma within a year at low frequency.

PDX-1-Cre, LSL-Kras$^{G12D/+}$, LSL-Trp53$^{R172H/+}$ (KPC) transgenic model

In the same previous study, Hingaroni *et al.* (2005) generated a conditionally expressed point mutant allele of the frequent mutant version of Trp53 (LSL-Trp53$^{R172H/+}$) and crossed it to the KC mouse. This leads to the conditional activation of both Kras^{G12D} and the Trp53^{R172H} alleles in the pancreas of transgenic animals, driven by PDX-1-Cre (KPC). In these mice, early PanIN lesions are observed between 4 to 6 weeks old mice. Once again, these animals display the whole range of PainIN lesions, but in addition, they develop high disease burden by 10 weeks at the earliest. The KPC animals have a significantly shortened median survival (~5 months) compared to wild type, PC and KC animals.

Pancreatic cancer mouse modeling using retrograde viral vector delivery:

Despite the fact that conventional genetically engineered mouse models of human PDAC have been helpful in understanding the disease's progression, these models are far too time-consuming, expensive, and labor-intensive. This complicates the ability to perform the extensive molecular studies needed to fully understand the disease. To circumvent these issues, several strategies have been developed, such as the direct intrapancreatic delivery of recombinant virus harboring constructs to manipulate genes of interest to rapidly model different molecular aspects of PDAC

(Chiou *et al.*, 2015). Even though these models require sophisticated transgenic and surgical skills, they are ideal to answer certain questions.

Chiou *et al.* (2015) demonstrated that retrograde pancreatic ductal injections of adenoviruses or lentivirus constructs containing Cre-recombinase leads to PDAC initiation and progression to metastasis in the current KP mouse models. This eliminates the need for the transgenic Cre alleles seen in traditional GEMMs of pancreatic cancer models and leads to more controlled, rather than widespread expression of oncogenic Kras and deletion of tumor suppressor genes. This model more closely recapitulates the human disease progression.

The ability to develop PDAC in Cre-lox mouse models without requiring the use of a transgenic Cre alleles speeds up and lowers the cost of molecular research in pancreatic cancer. This method is also compatible with the use of CRISPR/Cas9 gene inactivations in the pancreas. In fact, Chiou *et al.* (2015) integrated a Cre-conditional Cas9 allele into the K mouse model, and used lentivirus containing Cre-recombinase and sgRNA to inactivate other tumor suppressor genes. Interestingly, targeting of Lkb1 in combination with oncogenic Kras lead to rapid tumor progression and selection of Lkb1 knockout tumors, that resembled the Cre-mediatias Lkb1 loss allele. This technique revolutionized our ability to quickly investigate how genes functioned as this cancer developed.

1.3.2 Organoids

The term organoid (organ-like) was used for the first time in 1946 (Smith and Cochrane, 1946), however, it was with the establishment of intestine organoids by Sato

et al. in 2009 that the field significantly expanded. Nowadays, it is possible to isolate primary and tumor organoids from many tissues including the colon, pancreas, and lungs. An organoid is a 3D cellular structure that has the same identity of the organ it is intended to model, the diversity of cell types found in the organ, the functions unique to that organ, and the self-organization of the tissue that it should model (Frappart and Hofmann, 2020). Organoids can be easily characterized molecularly, tested in drug screens, and cultured indefinitely.

Recent studies have shown the ability to culture normal and neoplastic cells from the pancreatic epithelium of both human and mice (Boj *et al.,* 2015; Huch *et al.*, 2013; Hindley, 2016). In humans, these organoids can be established fine needle aspirates taken from PDAC patients with advanced disease, allowing for therapeutic testing and monitoring of tumor response to treatment. By embedding cells in Matrigel, it is possible to recapitulate the microenvironment of the tissue, which is a requirement for organoid growth. All critical elements of the basement membrane are present in Matrigel, which is also supplemented with medium with the bare minimum requirements for pancreatic epithelial cells to proliferate sustainably (Miquel et al., 2021). In addition, current culture conditions prevent normal ductal cells from quickly exhausting in vitro and upon orthotropic transplantation they create a proper ductal architecture (Boj *et al.,*2015). The ability to transplant neoplastic organoids into mice has been shown to recapitulate the progression of the disease from PanINs to metastatic PDAC (Figure 5) (Boj *et al.,*2015), thereby providing a tractable and transplantable system to test molecular and cellular properties of PDAC tumor progression.

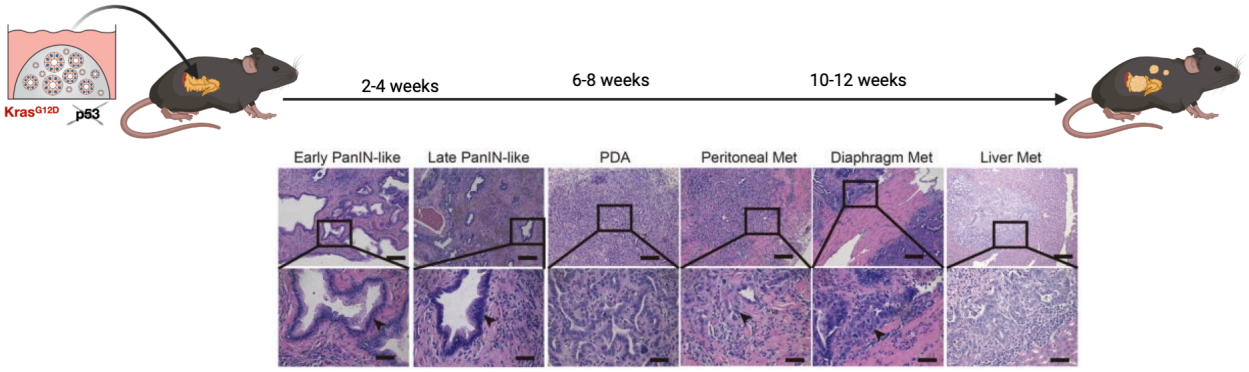


Figure 5: PDAC tumor progression upon orthotopic transplantation or KP organoids. Organoids harboring oncogenic Kras^{G12D} and deletion of tumor suppressor Trp53, upon orthotopic transplantation, recapitulate the tumor progression of the human disease from early pancreatic intraepithelial neoplasias (PanINs) to adenocarcinoma (PDA) and lastly to metastasis (Mets). Adapted from Boj *et al.* (2015).

These results support the fact that organoids should be one of the main focus of PDAC research in the future. Organoids are still a complicated model that needs extensive technical knowledge and is expensive to create and maintain. Although organoids display similarities with patient transcriptomics subtypes and chemoresistance profiles, it has been demonstrated that their transcriptomes change during ex vivo passage, which may limit their prognostic capacities (Miquel et al., 2021), therefore proper passage monitoring is necessary. Further work on accelerating organoid establishment and testing methodologies of important drugs is needed to bring quick organoid testing to the clinic for PDAC patients.

1.4 Advances in PDAC Metastasis Biology

The genetic progression of pancreatic cancer from a normal cell to pre-invasive lesions (PanINs), all the way to invasive PDAC is well defined. However, the molecular

mechanisms of the metastatic spread of PDAC remain largely understudied. Thus, there is still a need to uncover novel modulators of PDAC metastasis. Several groups have identified some genetic and epigenetic regulators of PDAC metastasis.

There have been a few studies that have evaluated naive primary tumor and metastases heterogeneity through whole-genome sequencing such as Yachida *et al* (2010), Campbell *et al* (2010), Sanborn *et al* (2015) and Hoogstraat *et al* (2014). Other studies, have looked at metastatic lesions after therapy, however, most of the time the genetic alterations found are a reflection of the therapeutic agent, which leads to mutagenesis, high selective pressures and bottlenecks. Therefore, this studies do not look at the disease's nature genetic progression. On the other hand, Makohon-Moore *et al* (2018) performed whole genome sequencing of genomic DNA from 39 samples (26 metastatic lesions, up to 3 distinct regions of each primary tumor and normal tissues) across four patients with metastatic PDAC. Here, the authors found that the vast majority of the mutations found were found in all the samples of the same patient. All patients shared KRAS mutations, as well as mutations in other known driver genes such as TP53, SMAD4, ARID1A, and ATM. However, unique mutations were still identified in the in the primary tumors, but none of these were found to be metastasis specific.

Furthermore, acquired molecular changes can promote cancer progression to metastasis. Chiou *et al* (2017) used genetically engineered mouse models (GEMMs) of PDAC and uncovered a transient subpopulation of cancer cells with high metastatic potential. The authors further identified the transcription factor BLIMP1 as a driver of PDAC metastasis leading to an enrichment of hypoxia-associated gene expression

programs. These findings show that upregulation of BLIMP1 links changes in the tumor microenvironment to a more aggressive and metastatic cell population. In addition, Whittle *et al* (2015) identified that the transcription factor RUNX3 controls a metastatic switch in PDAC using GEMMs. The authors of this study show that RUNX3 acts as both a tumor suppressor since it slows down proliferation, but also as a promoter since it orchestrates a metastatic program that promotes cell migration, invasion and adaptation to foreign organ colonization. Lastly, Maddipati *et al* (2021) used a multi-fluorescenct lineage labeled mouse model of PDAC to track paired primary and metastatic tumors. The authors noticed that in mice with multiple color primary tumor, metastasis to the liver and lung were from a single tumor clone, suggesting that tumor cell intrinsic factors also influence metastatic potential of a tumor. To understand the mechanism, the authors examined primary tumors from different colors where mets were attributed to just one color by performing DNA copy number analysis, RNA sequencing and functional studies. Copy number analysis showed unique DNA copy number profiles in different colored tumors, indicating that they arose independently, but similar copy number profiles were observed within the primary and metastases of a single color which is consistent with previous studies. Furthermore, this study showed that highly metastatic tumors had amplifications of Myc more frequently than low metastatic tumors, which were maintained in the paired metastases. Genomic and transcriptomic analysis revealed that high metastatic burden was associated with gene amplification and/or transcriptional upregulation of MYC and its downstream targets.

In addition, studies have identified epigenetic modulators of PDAC metastasis. Roe *et al* (2017) shows that metastatic progression of PDAC is associated with large-

scale enhancer landscape reprogramming by FOXA1, which activates early endodermal stem cells transcriptional programs.These metastatic lesions may go through a retrograde developmental transition because such reprogramming activates genes linked to foregut endoderm development. To further validate these findings, the authors profiled genome-wide enrichment of H3K27ac, an epigenetic mark associated with higher transcription and therefore defined as an active enhancer mark, in organoid cultures derived from normal pancreatic ducts, PanIN lesions, and primary and metastatic tumors. Even though there were no changes in the global level of H3K27ac between all samples, there were regions identified with increased or decreased H3K27ac, referred as GAIN and LOSS regions, respectively. GAIN regions were enriched for motifs such as binding sites for the AP1, SOX, and Forkhead families of transcription factors. When looking more closely at the Forehead family member FOXA1, since it was found to be unregulated in metastatic organoids, the authors identified a GAIN enhancer at the FOXA1 locus in metastatic organoids. Lastly, the authors found that in GEMMs of PDAC there are sporadic regions of focal Foxa1 upregulation in primary PDAC tumors, which becomes homogeneously unregulated in metastatic lesions.

Despite the clinical importance of metastatic spread, we still have a limited understanding of the molecular mechanisms that drive metastasis. This prompted me to question other mechanisms that may impact PDAC metastatic progression, such as regulation of cellular processes at the protein abundance and stability level.

PART 2: Intracellular Protein Degradation

2.1 Introduction

Protein turnover is a concept that has only been around for about 80 years. It was thought that protein components in the body were stable and did not change much, while proteins from diet acted independently as energy-providing fuel. This concept was challenged by Rudolf Scheonheimer who showed that protein components in the body are significantly turning over by tracking ^{15}N-labeled amino acids (Scheonheimer, 1942). Later, in 1946, early studies on nitrogen balance conducted by Benedict, Colin, Gamble, Smith, and others reveal that the amount of protein in the diet affects how quickly proteins are broken down (Peters and Van Slyke, 1946).

However, until the mid-1950s, the notion that proteins were turning over was not widely accepted. An example of this was seen when Hogness and colleagues (1955) examined the kinetics of β-galactosidase in *Escherichia coli and stated: "To sum up: there seems to be no conclusive evidence that the protein molecules within the cells of mammalian tissues are in a dynamic state. Moreover, our experiments have shown that the proteins of growing E. coli are static. Therefore it seems necessary to conclude that the synthesis and maintenance of proteins within growing cells is not necessarily or inherently associated with a 'dynamic state'*."

Around that time, Melvin Simpson showed that intracellular proteolysis in mammalian cells requires energy, which opened the field to look at insights into the mechanisms or metabolic logic of this observation. Simpson (1953) studied the release of amino acids from protein by isotopic labeling of proteins from rat livers with

methionine-S^{35}. This work demonstrated that conditions that restrict the release and consumption of energy, such as anaerobiosis, cyanide, or dinitrophenol, reduced the release of tagged methionine and leucine from rat liver slices. The peptide bond hydrolysis is an exergonic process thus there is no thermodynamic reason to use energy for this. As a result, the seeming need for energy indicated that something was poorly understood. When Goldberg's group demonstrated that aberrant proteins were quickly eliminated from the cell in the middle of the 1970s, part of the answer started to become clear (Goldberg and Dice, 1974; Goldberg and John, 1976; Schimke, 1976). According to Goldberg and Schimke, enzymes that catalyzed rate-limiting steps in metabolic pathways were often short-lived, and their quantities were responsive to metabolic changes (Schimke, 1976). As a result, during the late 1970s, researchers started to wonder whether the energy dependency of intracellular proteolysis was due to an energy-dependent regulation of proteolytic systems.

These studies served as the foundation for the intracellular proteolysis field. The potential for identifying the mechanics of protein turnover was opened by the understanding that proteins do really undergo significant turnover, that this process is particular, and that the stability of numerous proteins is regulated individually and can vary under various conditions.

2.2 The Lysosome and Autophagy

The discovery of the lysosome was a turning point in the field of protein degradation. Multiple studies had established that cellular proteins are constantly being synthesized and degraded (Scheonheimer, 1942; Peter and Van Slyke, 1946; Simpson,

1953), so the simultaneous discovery of an organelle containing a diverse array of secluded proteases with different specificities provided, for the first time, a mechanism that could possibly mediate intracellular proteolysis.

This organelle, called lysosome, was first identified in rat livers as a vacuolar structure that optimally functions at an acidic pH and encapsulates many hydrolytic enzymes. It is surrounded by a membrane that proteins the rest of the cell components from the enzyme's activity (De Duve *et al.*, 1953; Gianetto and De Duve, 1955). De Duve and his team discovered that the glucose-6-phosphatase precipitated irreversibly at an acidic pH, which led de Duve to speculate that the enzyme might be connected to agglutinated cytoplasmic membranes. After careful examination of cellular fractions using Claude's method, de Duve's group developed a procedure to isolate most of the active acid phosphate, and what the group in fact did was the purification of a new organelle relying solely on biochemical experiments. De Duve also identified four other acid hydrolyses in the fraction (β-glucuronidase, cathepsin D, ribonuclease, and DNAse), which lead them to formulate the following "lysosome" concept: "a membrane-bound organelle that contains hydrolyses with various specificities and whose main function is the intracellular digestion of macromolecules". This discovery, lead to the Nobel Prize in 1974 to Christian De Duve, Albert Claude, and George Palade "for their discoveries concerning the structural and functional organization of the cell.

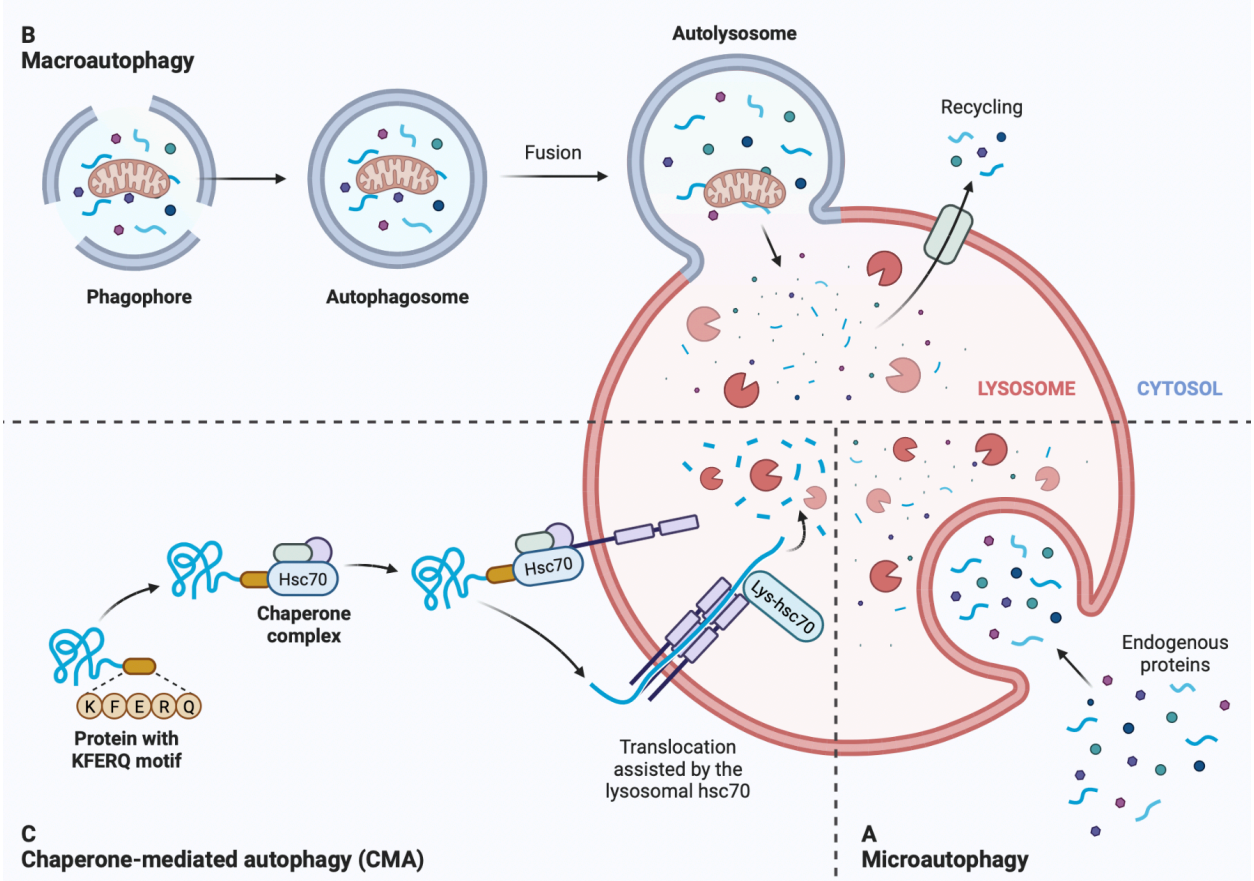


Figure 6: **Main types of autophagy**. The figure shows the main digestive processes that are mediated by the lysosome: the engulfment of **(A)** intracellular proteins (microautophagy) and **(B)** organelles (macroautophagy); **(C)** chaperone mediated autophagy by the recognition of the KFERQ motif. Modified from "Three Main Types of Autophagy" template, by BioRender.com (2022). Retrieved from https://app.biorender.com/biorender-templates.

The basic functional mechanism of the lysosome, known as microautophagy, occurs during basal metabolic conditions, where parts of the cytoplasm with entire cellular proteins are separated in the membrane-bound compartment that then fuses with a nascent lysosome leading to the digestion of the proteins (Ciechanover, 2005) (Figure 6A). Moreover, other organelles in the cell such as the mitochondria, endoplasmic reticulum membranes, glycogen bodies and others are engulfed under stress conditions, in a process known as macroautophagy (Ciechanover, 2005) (Figure

6B). However, these non-selective processes could not reconcile the emerging notion that different proteins are degraded with distinct half-lives in a process that is affected by pathophysiological conditions such as nutrient and hormone deprivation. Interestingly, in 1986, a study by Dice et al. indicated that lysosomal degradation also happens when a defined motif (KFERQ) is recognized in a protein by the HSC70 chaperone complex (chaperone-assisted autophagy), which leads to the translocation of the target protein into the lysosome (Figure 6C). The possibility of such a mechanism being substrate-specific was ruled out by the presence of a similar motif in about 30% of cellular proteins.

The development of methods to monitor protein kinetics in cells, as well as specific and general lysosomal inhibitors, lead to the identification of long- and short-lived cellular proteins. Poole and colleagues in 1977 and 1978 ‘fed’ ^{4}C-leucine-labeled dead macrophages to ^{3}H-leucine-labeled living macrophages, which allowed the monitoring of protein digestion within the same cell but from different sources: from within the cell (^{3}H-leucine) and from the extracellular milieu (^{14}C-leucine). The authors then treated the cells with compounds that raise the intralysosomal pH which inhibits the proteases in the lysosome. They found that these agents specifically inhibited the degradation of extracellular proteins, which lead the authors to predict the existence of non-lysosomal proteolytic systems that degrade intracellular proteins.+

As previously stated, the lysosome's functional mechanism could not explain many emerging protein degradation concepts such as the influence of nutrients and hormones, the differential effects of selective inhibitors, and the dependence on metabolic energy. Because proteolysis is an exergonic process, the metabolic energy

requirement for protein degradation was believed to happen upstream of protein breakdown. Hershko and Tomkins (1971) noticed in their studies that after ATP depletion, the activity of tyrosine amino-transferase was stabilized, indicating that energy might be required early in the proteolytic pathway. In light of the discovery of lysosomes in eukaryotic cells, it was suggested that energy is needed for substrate transport into the lysosome or for maintaining the low intralysosomal pH. However, the same phenomenon was observed in bacteria, which lack lysosomes, suggesting that energy is required for the regulation of the proteolytic process in both prokaryotes and eukaryotes (Goldberg and Etlinger, 1977).

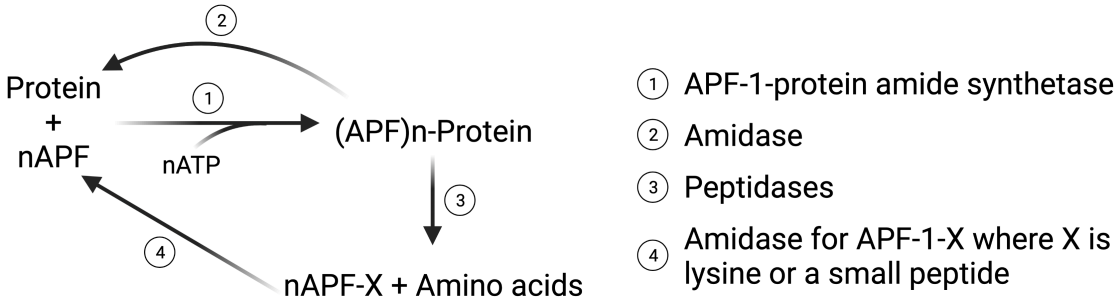
Progress in identifying the putative non-lysosomal proteolytic systems was hindered by the lack of a cell-free assay that recapitulated the other cellular proteolytic events in a specific and energy-dependent manner. Rabinovitz and Fisher (1964) made a significant discovery when they found that rabbit reticulocytes efficiently degrade abnormal hemoglobin containing aminoacid analogues. Because reticulocytes are immature, terminally differentiating red blood cells that lack lysosomes, it was hypothesized that hemoglobin degradation is mediated by a non-lysosomal machinery. Etlinger and Goldberg (1977) were the first to isolate a reticulocyte-derived cell-free proteolytic preparation. The crude extract required ATP hydrolysis and preferentially degraded aberrant hemoglobin, and performed best at neutral pH, indicating that the proteolytic activity was not lysosomal. Hershko, Ciechanover, and colleagues isolated and characterized a similar system shortly after, and later resolved, characterized, and purified its components — an accomplishment that resulted in the discovery of the ubiquitin signaling system.

2.3 Ubiquitin - Proteasome Pathway

Avram Hershko, Aaron Ciechanover, and Irwin A. Rose discovered and described the ATP-dependent, ubiquitin-mediated protein degradation pathway in a series of ground-breaking biochemical investigations in the late 1970s and early 1980s, leading to the Nobel Prize of Chemistry in 2004.

An essential source for the purification and identification of the enzymes involved in the ubiquitin-proteasome system was the cell-free proteolytic system from reticulocytes. The reticulocyte lysate was initially processed via a column of diethylaminoethyl cellulose in 1978 to remove hemoglobin (Ciechanover et al., 1978). This divided the lysate into two portions that, when combined, restored ATP-dependent proteolysis but were individually inert. Additional system enzymes were discovered as a result of this biochemical complementation strategy, all of which are necessary to catalyze the multistep process for the degradation of substrates. This was a significant and valuable observation that lead to the future discovery of the different components of the proteolytic system, since it showed that this process is not made up of a single classic protease, but rather it had multiple components. After careful examination of the fractions, the active component of the first reaction was a heat-stable protein called APF-1 (active principle of fraction 1) (molecular weight ~9000). Wilkinson, Urban, and Haas later identified this protein as ubiquitin (1980). The following year, Hershko, Ciechanover, and Rose used salt precipitation to further separate the second fraction of the reticulocyte lysate leading to the identification of an ATP-stabilized protein of ~450kDa (likely the protease but this was not further studied for almost 10 years), and the E1-E3 enzymes which were later isolated (Hershko, Ciechanover and Rose, 1979).

Up to this point, the role of APF-1 was unknown. A potential hypothesis was that APF-1 could be an activator for a protease in the second fraction. A breakthrough came in 1980, described in two seminal papers presented at the National academy of Sciences of the USA. Unexpectedly, the findings of the first study showed that a number of the proteins in the lysate were covalently bound by ^{125}I-labeled APF-1 when combined with the second fraction, which requires ATP (Ciechanover et al., 1980). In the second paper, the APF-1 protein was not labeled, but rather three substrates were labeled (lysozyme, α-lactalbumin and globin), which led to the discovery that several APF-1 polypeptides might be conjugated to the same substrate protein (Hershko et al., 1980). Treatment resistance to hydroxyl amine and alkali suggested an amide link via the lysine's ε-amino groups. A deubiquitinating enzyme activity that could liberate conjugated APF-1 from substrate molecules was also present in the lysate. As a result, an APF-1-protein amide synthetase and an amidase were found to be two new enzymatic activities. Following these discoveries, a graphic illustrating the proposed timeline of events in ATP-dependent protein breakdown was put out (Hershko et al., 1980).



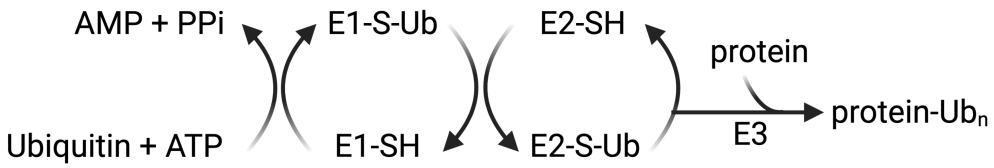
Amino-acid analysis of APF-1, its molecular mass and other general characteristics, revealed that, in fact, APF-1 is ubiquitin (Wilkinson, Urban, and Haas,

1980), a known protein with a previously unknown function. This discovery and the finding that APF-1 attachment to the substrate is similar to the one that connects ubiquitin to histone H2A, solved the mystery of the energy requirement for intracellular proteolysis. Wilkinson et al. (1980) proposed that ATP is required in an exothermic reaction because it allows for control and specificity, paving the way for researchers to better understand the complex mechanism of isopeptide bond formation. Between 1981 and 1983, Ciechanover, Hershko, and Rose tested the multi-step ubiquitin-tagging hypothesis by isolating and characterizing three distinct enzyme activities, E1, E2, and E3. The first enzyme that was identified and purified by the group was the ubiquitin-activating enzyme E1 (Ciechanover *et al.*, 1981). The group inhibited ATP-dependent proteolysis by treating the reticulocyte lysates with pyrophosphate, which revealed that the activation mechanism was an adenylyl-transfer at the carboxyl group of ubiquitin. As a result, ubiquitin rather than the substrate protein was the likely target of the activating process. In reality, the C-terminal glycine of ubiquitin was discovered to be the active amino acid residue through the reductive cleavage of the intermediate by tagged sodium borohydride (Hershko et al. 1981). This ubiquitin activating enzyme catalyzed a two step reaction. Firstly, the activation of the C-terminal carboxyl group of ubiquitin with the formation of an adenylate, which consumes ATP. Secondly, the adenylate is transferred to an acceptor sulfhydryl on the enzyme with the release of AMP (Figure 8) (Hershko et al. 1981; Haas et al, 1982). While these experiments were undergoing, a new covalent affinity chromatography method was developed (Ciechanover *et al.*, 1982; Hershko et al. 1981; Haas et al, 1982). The reticulocyte lysate fractions were passed through this sepharose column containing covalently attached

ubiquitin. In the presence of ATP, 50% of the ATP-pyrophosphate exchange activity was retained in the column, while in the absence of ATP no enzyme was bound. This lead to the purification of the activating enzyme, which was shown to be a homo-dimer of 210kDa. Additionally, the following purifications of the E2 and E3 enzymes relied heavily on this novel technique.

The realization that the ubiquitin activating enzyme E1 could not form ubiquitin-protein conjugates by itself lead the group to use this new purification method to isolate two further enzymes, E2 and E3 (Hershko *et al.,* 1983). Hershko and colleagues found E2 enzymes to have an apparent molecular weight of 35kDa and the E3 enzyme of 300kDa. The authors also found that in order for E2 to bind to the ubiquitin-sepharose column, it required the activity of E1 and ATP, while the binding of E3 did not require any of these. Together, the findings showed that E2 bound to the column covalently, just like E1, while E3 did not. The iodoacetamide-induced suppression of E2 activity, which could be avoided by preincubating with E1 together with ubiquitin and ATP, suggested that E2 had an iodoacetamide-sensitive thiol site. The idea that E1 might transfer ubiquitin to E2 and that E2 might assist in the transfer of ubiquitin from E1 to the substrate was now put to the test. Furthermore, during SDS-polyacrylamide gel electrophoresis, the thiol ester of E1-ubiquitin did not dissociate, and its stability allowed Hershko and colleagues (1983) to look for the transfer of ^{125}I-ubiquitin from E1 to E2 on gels. While there was evidence of ubiquitin transfer from E1-ubiquitin to E2 enzymes, there was no evidence of transfer from E1-ubiquitin to E3. Instead, E3 aided in the formation of amide bonds as it catalyzed the transfer of labeled ubiquitin from E2 to the protein substrate. The full conjugation pathway was now clear: activated

ubiquitin bound to the thiol ester site of E1 with its COOH terminus, then ubiquitin is transferred to a sulfhydryl site on E2 enzymes, and lastly, ubiquitin is transferred to firm stable protein conjugates in the presence of E3 enzymes, leading to polyubiquitination of the target protein (**Figure 8**):



The discovery of the downstream protease that would specifically detect ubiquitinated substrates was the final missing piece at this point. Tanaka and colleagues (1983) discovered a second ATP-demanding step in the reticulocyte proteolytic system after ubiquitin conjugation, and Hershko and colleagues (1984) demonstrated that energy is required for conjugate destruction. Later, Hough and colleagues (1986) made a significant contribution to the field by discovering a high-molecular-mass alkaline protease that destroyed ubiquitin-tagged but not untagged lysozyme in an ATP-dependent manner (Figure 7). Waxman and colleagues (1987) discovered that the protease is an unusually large enzyme with a molecular weight of 1.5MDa, which was later confirmed. In addition to the larger 26S complex, a smaller, neutral, multisubunit 20S protease complex was discovered (Hough et al., 1987). This 20S protease is ATP-independent and has several distinct catalytic activities, including cleavage on the C-terminus of hydrophobic, basic, and acidic residues, raising the possibility that it is part of the larger 26S protease that degrades ubiquitin conjugates (Hough et al., 1987). Later research revealed that the 20S complex is, in fact, the core catalytic particle of the larger 26S complex (Eytan et al., 1989; Driscoll and Goldberg, 1990). Hoffman and colleagues (1992) mixed the two purified particles to create the

active 26S enzyme (later known as the 26S proteasome) and provided direct proof that the assembly of a 19S ball-shaped regulatory subcomplex and the catalytic 20S core results in the mushroom-shaped 26S protease.

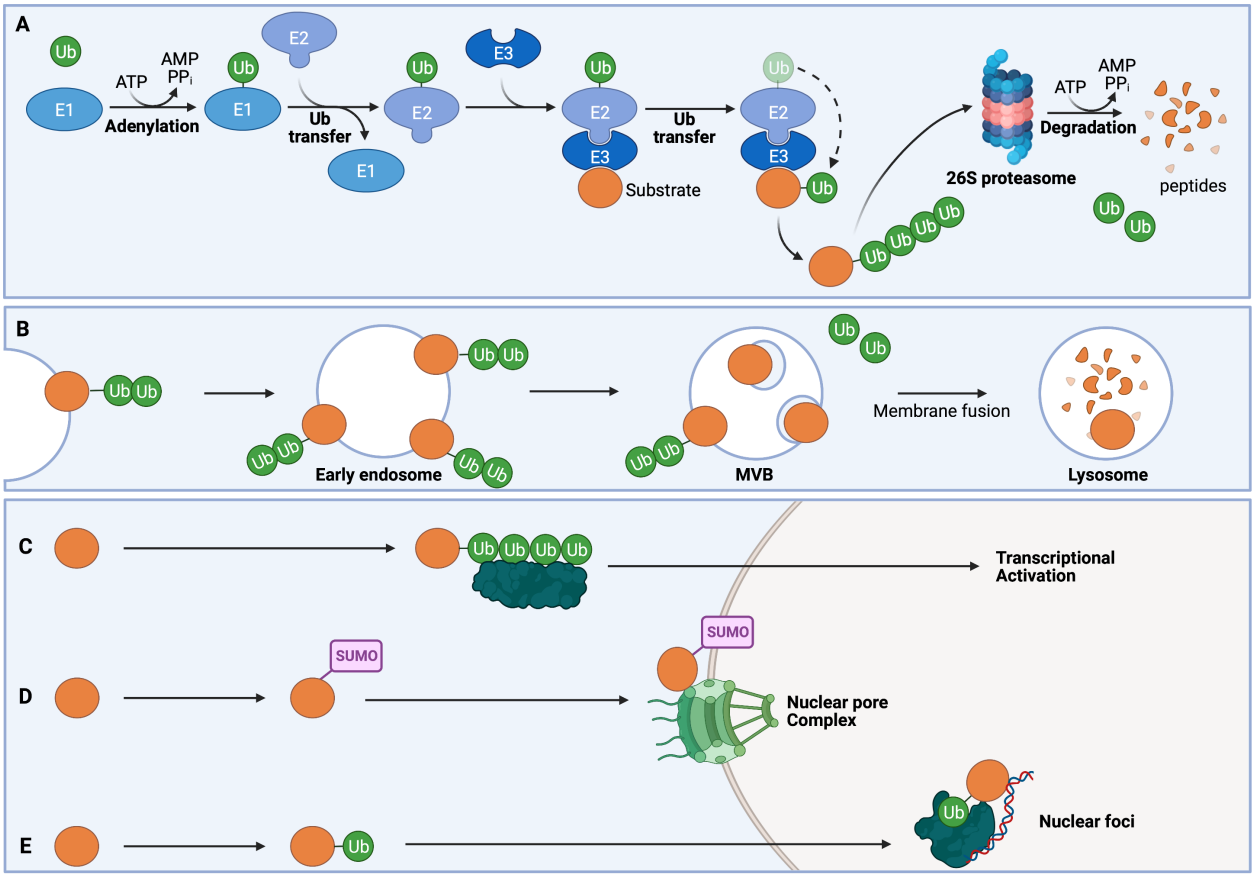


Figure 7: Various functions of ubiquitin and ubiquitin-like modifications. (A) Polyubiquitnation of substrate proteins by the sequential enzymatic activity of E1, E2 and E3 enzymes leading to proteasomal degradation of substrate. **(B)** Mono- or oligo-ubiquitination of membrane proteins leading to lysosomal degradation. **(C)** Polyubiqtionatopn can also lead to the activation of transcriptional regulators. **(D)** Monoubiquitination or **(E)** SUMOylation can localize proteins to different subcellular destinations such as the nuclear pore complex and nuclear foci, respectively. MVB, multivesibular bodies; Ub, ubiquitin; Pi, inorganic phosphate. Adapted from Ciechanover, 2005. Created with BioRender.com (2022).

Proteasomes can be found in both the nucleus and the cytoplasm. The barrel-shaped 20S structure protects the proteasome's active sites, by shielding them from

the cellular environment. Poly-ubiquitinated proteins are recognized by the regulatory 19S complexes of the proteasome, which further unfold them and help move them through a small gate into the 20S core particle, where degradation takes place, leaving only peptides with 7-9 amino acid residues. The 19S complex's isopeptidase then extracts ubiquitin from the substrate protein and recycles it for use in other ubiquitination reactions.

The understanding that regulated proteolysis is involved in regulating a wide range of cellular processes, including the cell cycle and cell division, apoptosis, transcription, antigen presentation, signal transduction, receptor-mediated endocytosis, protein quality control, and the modulation of various metabolic pathways, was another significant development that followed the discovery of the ubiquitin-proteasome system (Figure 7). As a result, intracellular proteolysis was elevated from a disregarded process and research area to a crucial subject in contemporary biology.

2.3.1 Ubiquitin Activating (E1) Enzymes

The human genome encodes two ubiquitin-activating enzymes (E1s) that activate ubiquitin (Ub) and transfers them to ~40 ubiquitin-conjugating enzymes (E2s). The general mechanism of the E1-catalyzed reaction was well established by Haas and Rose (1982), Haas *et al.* (1982) and Hershko *et al.* (1983). In summary (Figure 8), E1 first binds to Ub in an ATP-dependent manner and catalyzes the adenylation of the Ub C-terminus. Second, between a conserved catalytic cysteine and the Ub, E1 forms a thioester. E1 is then loaded with a second Ub molecule, and its C-terminus is

adenylated. Finally, the ternary E1-Ub thioester complex recruits an E2 to facilitate thioester-linked Ub transfer to a conserved E2 cysteine (transthioesterification).

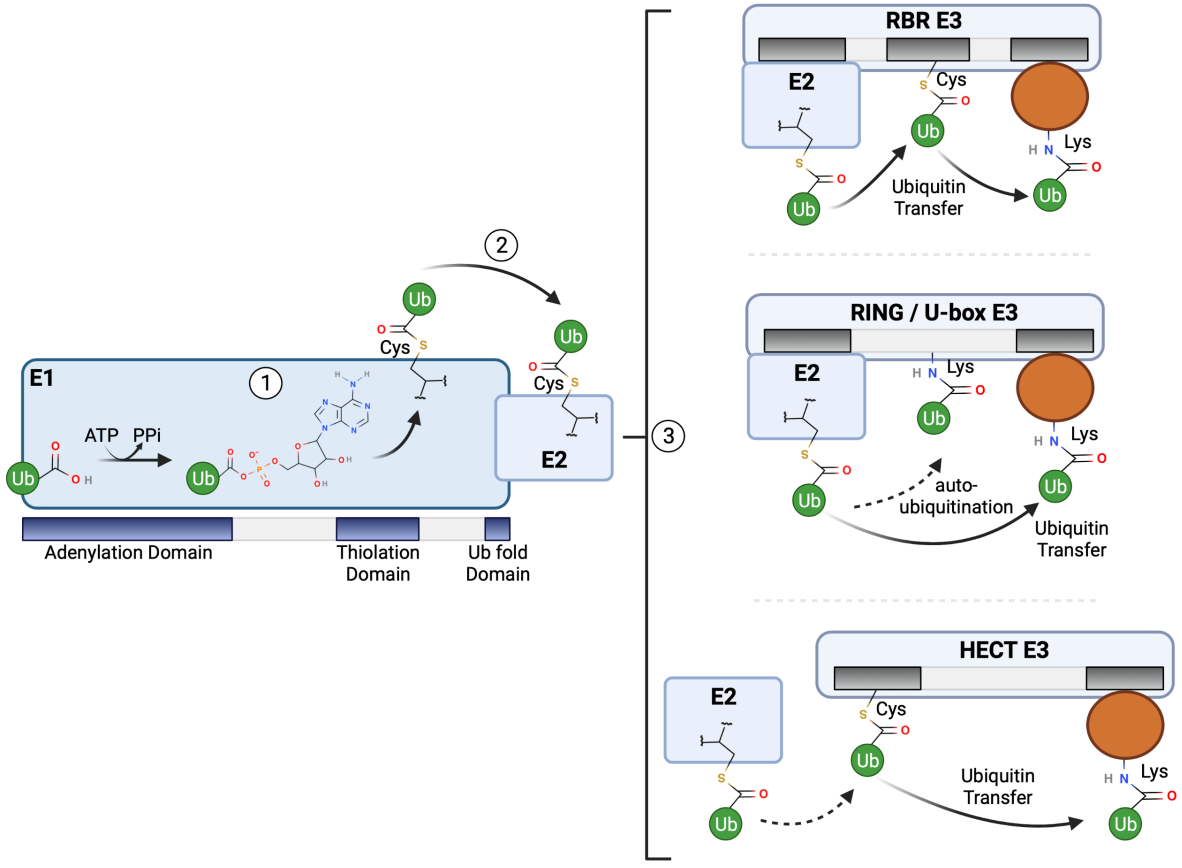


Figure 8: The ubiquitin-conjugating system. **(1)** Ubiquitin activating (E1) enzymes first activate the C-terminal carboxyl group of ubiquitin with the formation of an adenylate, and secondly, the adenylate is transferred to an acceptor sulfhydryl on E1. **(2)** The E1-Ub thioester complex recruits an Ubiquitin Conjugating (E2) enzyme to facilitate thioester-linked Ub transfer to a conserved cysteine (transthioesterification). **(3)** Ub is conjugated to lysine-amino groups either directly or through complexes catalyzed by ubiquitin ligase (E3) enzymes using the energy stored in the E2-Ub thioester. The RING1 domain binds to E2-Ub and transfers ubiquitin to the RING2 domain, which then conjugates ubiquitin to the substrate. This structure is known as RING1-in-between-RING (IBR)-RING2 (RBR). RING/U-box E3s can function alone or as a component of multisubunit complexes and depend on E2s to ubiquitinate substrates. HECT E3s conjugate ubiquitin to the substrate by forming a thiol-ester bond with it. Created with BioRender.com (2022).

The major E1 enzyme in yeast is the *Saccharomyces cerevisiae* (Uba1) enzyme, which shares extensive homology with the human orthodox Ube1 and both adopt the same architecture and activity. The Uba1-Ub structure consists of a complex arrangement of six structural domains:

1. The adenylation domains that have MoeB/ThiF-homology motifs, are referred as inactive and active motifs (IAD and AAD, respectively). The AAD is the domain that binds ATP and Ub (Lee and Schindelin, 2008; Lake et al., 2001; Lois and Lima, 2005; Walden et al., 2003)

2. Two catalytic cysteine half domains inside the adenylation domains, that have the E1 active site cysteine (FHHC and SCCH, respectively) (Szczepanowski et al., 2005; Lee and Schindelin, 2008)

3. The four-helix bundle (4HB) is part of the IAD, and immediately follows the FCCH

4. The C-terminal ubiquitin-fold domain (UFD), which recruits E2s (Huang et al., 2005, Huang et al., 2007, Lois and Lima, 2005).

These domains cluster together to form a sizable central canyon, the end of which (near FCCH) successfully recruits ubiquitin molecules. Uba1 and Ub engage in three different ways: at the hydrophobic interface (Interface I), where the "canonical" hydrophobic patch of Ub interacts with the conserved AAD of Uba1, at the polar interface (Interface II), and at the polar interface (Interface III), where Ub interacts with the FCCH (Lee and Schindelin, 2008). The SCCH domain has the E1 catalytic cysteine that joins with the C-terminus of Ub to generate the thioester and facilitates the transfer

of Ub to its E2. The thioester complex between Uba1 and Ub is formed by the E1 active site cysteine nucleophilic attack on the Ub~adenylate, leading to the deprotonation of the E1's cysteine by a general bae catalyst (Lee and Schindelin, 2008). In addition, the active site cysteine has to be close to the Ub C-terminus in order to form the E1-Ub thioester. However, this isn't the case, which strongly implies that the complex would need to undergo major conformational modifications in order to accommodate the juxtaposition of the bound Ub-adenylate and the active site cysteine thiol. To accomplish this, the C-terminal flexible tail of ubiquitin moves in hinge motions, changing its relative position to the AAD, SCCH and FCCH domains, and also it performs other conformational changes around the catalytic cysteine in the SCCH (Walden et al., 2003; Lee and Schindelin, 2008). Additionally, following the creation of the thioester linkage, the catalytic cysteine must retract with the developing Ub thioester in order to permit the adenylation of a second Ub molecule.

The primary role of E1 is the transfer of activated ubiquitin to the associated E2 enzyme. In this scenario, one of the E2 enzymes and E1 interact noncovalently, and then, in a subsequent transthioesterification step, the Ub is transferred from E1's catalytic cysteine to that of E2's. Lee and Schindelin (2008) found that E2s bind on the opposite side of and face away from E1's catalytic cysteine, meaning that significant conformational changes were needed to enable E1 and E2 catalytic cysteines to approach each other for the transfer of Ub. In fact, Uba1 detailed crystal structure showed that the E1-Ub conjugate undergoes distinct conformation changes to allow this to happen (Lee and Schindelin, 2008). It's interesting to note that compared to other E1s, Uba1 has a much wider canyon between its SCCH and UFD, which may

enable it to accommodate a variety of E2 partners, some of which have different N- and C-terminal extensions outside the E2 core domain. Additionally, the UFD appears poised in the unobstructed conformation and ready to accept available E2s. As a result, the expanded UFD linker connects the UFD to the adenylation domain, and a slight modification at the hinge causes a significant change in the orientation of the entire UFD.Once both catalytic cysteines are in close proximity, a transthioesterification reaction takes place producing an E2-Ub conjugate.

2.3.2 Ubiquitin Conjugating (E2) Enzymes

Humans have ~40 E2s that are involved in the transfer of ubiquitin and ubiquitin-like proteins. These enzymes carry out a variety of functional roles with just one active site, including transthiolation meaning canalizing transfer from a thioester to a thiol group; and aminolysis meaning transfer from a thioester to an amine group; as well as other less explored functions (Stewart et al., 2016). E2s interact with an E1 enzyme and one or more E3 enzymes, and in addition they may directly interact with target proteins determining where and how the target will be ubiquitinated (Figure 8).

Even though we know that E2 enzymes play important roles in the ubiquitination of target substrates, major questions remain in the field: why are E2 enzymes found in all eukaryotes, and why do we not directly transfer ubiquitin from E1 to E3? Potential hypotheses include that, while E3s primarily select the substrate, E2s determine the fate of the substrate, and that E2s promote ubiquitination independently of an E3 ligase, such as UBC22 (Sjoerd et al., 2009; Kraft et al., 2005).

All E2s possess a core catalytic domain, known as the UBC domain, that consists of ~150 amino acids and the active site's cysteine is needed for the formation of the thioester bonds. This domain consists of an α/β-fold, with four α-helices and a four stranded β-sheet, and important loop regions form part of the E3-binding site and the E2 active site (Stewart et al., 2016). The E3-binding site and the E2 active site both contain important loop regions. Most E2s have a single UBC domain, with multiple functionally distinct motifs. Interestingly, they can have short N- and C-terminal extensions that can impact the distinct specificity between E2s. These motifs have a conserved His-Pro-Asn tripeptide (HPN) at the 10th residue at the N-terminal of the cysteine residue (Liu et al., 2020). While the histidine residue is essential for the structure, the proline serves as a link to aspartic acid residue which catalyses the formation of the is-peptide bond. The E2 enzyme family is divided into four classes based on the location of the additional fragment in the UBC domain. Class I exists only in the UBC domain; class II exists in both the N-terminal and UBC domains; class III exists in both the C-terminal and UBC domains; and class IV exists in the N-terminal, C-terminal, and UBC domains.

The energy stored in the E2-Ub thioester is used to conjugate Ub to lysine-amino groups directly or via complexes mediated by E3s. Although there are topologically distinct classes of E3, some E2s function with multiple classes of E3. Most E2/E3 complexes have a moderate to poor binding affinity, with E3 attaching to the E2 UBC domain formed by the residues in helix 1, loop 4, and loop 7 (Stewart et al., 2020). The understanding of the characteristics of E2-Ub conjugates and the structural alterations that take place when they interact with an E3 and are prepared for

Ub transfer has advanced significantly, according to Stewart et al. (2020). Because of their considerable flexibility, the C-terminal residues of Ub (amino acids 72–76) enable an Ub molecule that is covalently attached to an E2 active site to swing by its tail and sample a variety of conformations with respect to the E2 domain. There are different orientations: one that involves little or no contact between the E2 and ubiquitin (“open states”) and one that involves contacts between Ub hydrophobic patch centered on Ub I44 and residues int he E2 crossover helix (“closed states”). Shifts towards populations of more “closed states” happens upon binding of an E3 ubiquitin ligase, dramatically enhancing the intrinsic reactivity of E2-Ub conjugates towards aminolysis and final polyubiquitination of target substrates.

Furthermore, E2s have been reported to play important roles in defining the linkage types of ubiquitin chains generated. Once the E2-Ub conjugate is formed, E2s orient a specific lysine of the acceptor ubiquitin to enable its approach and nucleophilic attack of the E2-Ub thioester, resulting in an isopeptide bond between the amino group of the acceptor ubiquitin and the C-terminal carboxylate of the donor ubiquitin, leading to a polyubiquitin chain (Zao *et al.,* 2020). However, it still remains a question in the field how the different E2s enzymes choose from the seven lysine residues in ubiquitin to orient for ubiquitination, which could lead to different ubiquitin chain topologies affecting downstream processing of the target substrate. Substrate specificity is brought by ubiquitin ligating (E3) enzymes which recognize the E2-Ub conjugate and the target substrate, and catalyze the transfer of the polyubiquitin chain to the target.

2.3.3 Ubiquitin Ligating (E3) Enzymes

Ubiquitin ligating (E3) enzymes bridge ubiquitin transfer from E2 to substrate proteins. The human genome encodes more than 600 E3s which are largely responsible for providing the exquisite substrate specificity that characterized the ubiquitin-proteasome system. According to differences in structure and function, E3 ubiquitin ligases can be divided into four types: HECT, U-box, RING-finger and RBR type (Figure 8). Direct ubiquitin transfer from E2-ubiquitin to the substrate is catalyzed by RING E3s. The catalytic cysteine in HECT and RBR E3s, in contrast, first accepts ubiquitin from E2 ubiquitin to generate an intermediate called an E3 ubiquitin thioester before transferring this ubiquitin to the substrate.

HECT E3 Ligases:

HECT (homologous to the E6-associated protein carboxyl terminus) E3 ligases is one of the largest and earliest studied E3 ligases. There are 28 HECT E3s in humans, which have an N-terminal substrate-binding domain and a C-terminal HECT domain (Rotin and Kumar, 2009). The HECT domain is ~350 amino acids and contains the catalytic components for ubiquitin conjugation and transfer. Due to differences in their N-terminal domains, HECT E3s can be further divided into three subfamilies: the Nedd4 family (9 members), the HERC family (6 members), and the HECTs (13 members) (Yang *et al.*, 2021). Need4 members are distinguished by WW and C2 domains that bind PY motifs in substrates. The N-terminal C2 domain can bind Ca^{2+} and phospholipid, which is required not only for protein targeting to phospholipid membranes, but also for substrate protein ubiquitination (Dunn et al., 2004; Tian et al.,

2011; Rizo and Sudhof, 1998). The HERC family members contain uncharacterized regulator of chromosome condensation 1-like (RDL) domains. RLDs have two major functions: they can act as a GEF to regulate the small GTPase Ran and interact with chromatin via histones H2A and H2B. (Zhang and Clarke, 2000; Nemergut et al., 2001). The HERC subfamily is further divided into two large and four small HERCs based on the number of RLDs (Yang et al., 2021). Finally, the HECTs family members have additional protein-protein interaction domains. HECT domains have two lobes joined by a flexible loop, with the N-terminal lobe containing catalytic cysteines and the C-terminal lobe containing the E2-Ub conjugate (Yang et al., 2021). The flexible appearance allows the lobes to rotate, facilitating ubiquitin transfer to the substrate (Verdecia et al., 2003). There are many other HECT E3 ligase members, such as E6AP and HUWEI. E6AP is the first HECT E3 discovered with a zinc-binding fold known as the AZUL (amino-terminal Zn-finger of Ube3a ligase) domain, which promotes K48-linkage polyubiquitination and substrate degradation (Yang et al., 2021). HUWE1, on the other hand, consists of a WWE domain and a ubiquitin-associated (UBA) domain, both of which regulate different aspects of cancer development (Yang et al., 2021).

Early structures of HECT E2 showed a wide gap between the catalytic cysteine residues and the E2 binding site, indicating that conformational modifications are necessary for the E2-E3 transthiolation reaction (Huang et al., 1999; Verdecia et al., 2003; Ogunjimi et al., 2005). These investigations showed that the HECT E3s' hinge rotates to place the C lobe's catalytic cysteine next to the E2-Ub bond, enabling transthiolation. The NEDD4 family of HECT E3s are more easily transthiolated due to hydrophobic interactions between ubiquitin and conserved residues on the C-lobe.

Further investigation is required to understand how these other HECT E3 interact with ubiquitin, since the C-lobe residues needed for successful transthiolation are nor conserved in these HECT E3s. In the NEDD4-Ub complex, the C-terminal tail of ubiquitin is held in an extended conformation by a hydrogen bond network made up of amides from the C-lobe of the HECT domain and backbone carbonyl oxygen moieties. According to the known E3-E2-Ub structural data, restricting the position of ubiquitin's C terminus appears to be a typical thioester-activating strategy for RING and HECT E3s (Maspero et al., 2013). A shared thioester-activating mechanism for RING and HECT E3s appears to be limiting the position of ubiquitin's C terminus.

After transthiolation, the E3-Ub thioester in HECT is put adjacent to a substrate lysine. This was first observed, in the crystal structure of HECT E3 Rsp5 using a three-way chemical cross-linker was used to bind the catalytic cysteine of Rsp5, the C terminus of ubiquitin, and a lysine side chain of a substrate protein (Kamadurai et al., 2013). In order to secure this conformation, the C-lobe rotates 130 degrees around the flexible linker, causing about half of the N-lobe to come into contact with the C-lobe. This brings the donor ubiquitin close to the substrate recognition domain allowing ubiquitin transfer to the substrate.

RING type E3 Ligases:

RING E3s fold their RING or U-box catalytic domain to allow for direct ubiquitin transfer from E2 to the substrate (Figure 8). According to bioinformatics studies, the human RING E3 family, which is the largest of the three E3 families, contains over 600 members (Yang et al., 2021). The RING domain is the only element that is necessary to

attract E2-Ub and to promote ubiquitin transfer. The following amino acid sequence typically makes up the RING finger protein: His-X2-Cys-X4-48-Cys-X2-Cys, where X is any amino acid; Cys-X2-Cys-X9-39-Cys-X1-3-His-X2-3-Cys (Deng et al., 2020). These RING domains are distinguished structurally by the presence of two zinc ions, which are required for RING domain folding, and are coordinated by Cysteine and Histidine residues organized in a cross-breed configuration (Yang et al., 2021).

The first crystal structure of the CBL-UBE2L3 RING E3-E2 complex showed that the active site of E2 is far from the E3 RING domain, ruling out direct involvement of the RING domain in catalysis (Zheng *et al.,* 2000). Later research claimed that the RING E3 binding caused an allosteric alteration in the E2 active site (Ozkan et al., 2005; Petroski and Deshaies, 2005). The E2-Ub conjugate is highly dynamic and flexible, and prefers to adopt conformations with little to no interactions between the E2 and ubiquitin. However, upon interaction with a RING E3 it shifts the equilibrium towards a closed conformation in which ubiquitin is proximal to the RING domain, priming the ubiquitin for transfer (Pruneda *et al.,* 2012). Even though the E2-Ub complex is primed by the RING domain alone, the presence of additional ubiquitin-binding components in the sequence of RING E3s aids in further stabilizing E2-Ub in the primed conformation and increases the enzymatic activity of the E3-E2-Ub complex (Pruneda et al., 2012). Priming the ubiquitin in the E2-Ub complex and juxtaposing a substrate lysine and the E2-Ub thioester are the primary functions of RING E3s in ubiquitin transfer. Both the target protein's structure and the carefully controlled structural restrictions of the E3-E2-Ub complex control whether a substrate lysine can be ubiquitinated.

Some E3s can attach to the substrate directly and catalyze its ubiquitination without the help of other proteins. One such E3 is Mdm2 (murine double minute 2) / Hdm2 (human enzyme) which directly promotes degradation of p53 (Wade *et al.,* 2013). However, some E3s' substrate recruitment and catalytic domains are made up of several proteins. For instance, the SCF (Skp1-Cullin1-F-box) complex, which comprises of the invariant Rbx1 (attract the E2 enzyme), Cul1 (scaffold protein), Skp1 (bridge F-box proteins (FBPs)), and a distinct FBP (harbor catalytic activity), is one such example (Skaar, Pagan and Pagano, 2014). A total of 70 FBPs have been found in humans, all of which act on substrate recognition in diverse biological processes that are downstream and are selectively regulated.

RBR type E3 ligases:

Approximately a dozen RBR E3s are encoded by the human genome, all of which are multidomain proteins of RING1, in-between RING (IBR) and RING2 domains (Figure 8) (Deng *et al.,* 2020). RING1 binds to E2-Ub conjugate and has the characteristics of RING-type E3s, whereas RING2 domains contain a catalytic cysteine nucleophile and has similar activity as HECT E3s. It forms a thioester bond intermediate with ubiquitin and transfers it to the substrate (Deng *et al.,* 2020). The RING1 domains harbor similar hydrophobic cores to canonical RING domains that interact with the loops of E2-Ub conjugates. However, they lack the ability to lock in the closed state which is primed for ubiquitin transfer to a lysine residue on substrate proteins (Wenzel *et al.,* 2011). Therefore, RBR E3s rely on E2s that preferably transfer ubiquitin to cysteines in order to transfer it to its RING2 domain. The RING1-IBR

module adopts an extended conformation in which two extension helices, hE1 and hE2, connect RING1 and IBR. Ub is put in a conformation for contact with a helix between the IBR and RING domains by the actions of hE2-IBR and RING1. This allows RING2 to bind the E2 and secure the C-terminal tail of ubiquitin in the expanded conformation (Lechtenberg et al., 2016). The E2 and RING2 catalytic cysteine residues are next to each other in this configuration, which places the ubiquitin in the best position for transfer.

Various crystal structures have shown distinct RING1-IBR-RING2 arrangements, including active confirmations allowing ubiquitin transfer and auto-inhibitory conformations that are not competent for ubiquitin transfer. This auto-inhibitory conformation maintains a large distance between the RING2 active side and E2 which decreases the activity of RBR by inhibiting the thiol-transfer reaction (Deng *et al.,* 2020). This is very important because aberrant activity of RBR E3s lead to a number of diseases including Parkinson;s disease and cancer.

2.3.4 Ubiquitin Family of Modifiers

The ubiquitin chain topology heavily influences the fate of ubiquitinated proteins. Three forms of ubiquitination linkage have been found based on structural characteristics: monoubiquitination, polyubiquitination, and branching ubiquitination (Figure 9). The attachment of a single ubiquitin to a specific lysine of the substrate, which has been implicated in the regulation of DNA damage repair, is referred to as monoubiquitination (Figure 7). An example is the E3 ligase Rad18, which recruits DNA polymerases to monoubiquitinate the proliferating cell nuclear antigen (PCNA) in

response to DNA damage repair (Gang et al., 2010). Other cellular activities mediated by monoubiquitination include autophagy and chromatin remodeling. Monoubiquitination of membrane proteins, for example, can influence their interactions with the autophagy adaptor protein p62, encouraging mitochondrial and peroxisome autophagy (Kwon and Ciechanover, 2017). Furthermore, lysine-specific monoubiquitination of histone, which is involved in chromatin remodeling, is a well-defined instance.

When more than two ubiquitin molecules are attached to the same lysine on a single substrate, this process is referred to as polyubiquitination. After the lysine residue is linked to ubiquitin, there are numerous types of polyubiquitination (K6, K11, K27, K29, K33, K48 and K63). As shown by the nuclear factor-B (NF-B) pathway, K63-linked or M1-linked ubiquitin chains control the assembly of signaling complexes, while K48-linked and K11-linked ubiquitin chains are typically involved with proteolysis (Figure 9). (Senft et al., 2018). A branching polyubiquitin chain comprises many linkage types that are also crucial for controlling a variety of cellular functions, as opposed to a homologous chain, which only has one kind of linkage. For instance, the Epsin1-mediated endocytosis of the major histocompatibility complex I (MHCI) involves the mixed K11 and K63 connections (Takahashi et al., 2018).

In addition to ubiquitin, there are also ubiquitin-like (UBL) proteins which include NEDD8, SUMO, FAT10, ISG15, ATG8, ATG1, HUB1 and FUB1 (Figure 9). These UBL proteins not only share sequence homology and structural similarity with Ub, but they also modify their substrate proteins via a similar enzymatic cascade. Two of the major UBL modifications are neddylation and SUMOylation. Neddylation refers to the

attachment of NEDD8, which has the highest homology with ubiquitin and regulates various biological processes in a dynamic and reversible manner (Deng *et al.,* 2020). Neddylation does not lead to degradation of the substrate, but rather leads to the activation of the substrates which subsequently control several biological processes such as cell cycle regulation and signal transduction. On the other hand, Small Ubiquitin-Related Modifier (SUMO) is linked to a substrate through the process of SUMOylation, which involves creating an isopeptide bond between SUMO's terminal glycine and the lysine of the substrate (Deng et al., 2020). SUMOylation has been associated with regulation of substrate localization, stability and activity.

Interestingly, each of these modifications are associated with specific E1 enzymes. The E1 for ubiquitin is a monomeric protein that weighs between 110 and 120 kDa, while the E1s for NEDD8 and SUMO are heterodimeric complexes with similar molecular weights (Lee and Schindelin, 2008). This brings high specificity to the ubiquitin-proteasome system.

Furthermore, the proteolytic activity of about 100 deubiquitylating enzymes (DUBs) also modifies the ubiquitin code, for example by removing K48-chains or K63-chains to stop signaling events or prevent destruction, respectively (Yau and Rape, 2016). Ubiquitin-specific proteases, ubiquitin carboxyl-terminal hydrolases, otubain proteases, Machado-Joseph disease protein domain proteases, JAMM/MPN domain-associated metallopeptidases, and monocyte chemotactic protein-induced proteins are six families that can be distinguished based on their structural and sequence. Except for the JAMM family of metalloproteinases, all of these DUBs are cysteine

proteases. These enzymes can attach to various types, topologies, or lengths of Ub chains and remove those chains from the substrate directly (Yau and Rape, 2016).

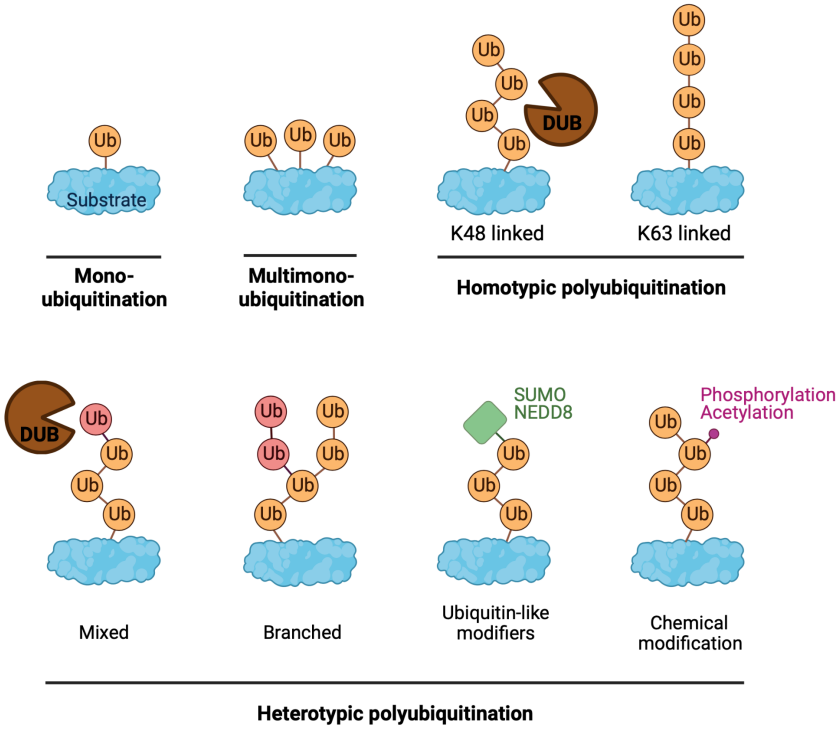


Figure 9: The Ubiquitin Code. Representative images of different ubiquitin and ubiquitin-like chain topologies. The different linkages were chosen arbitrarily. Modified from "The Ubiquitin Code" template, by BioRender.com (2022). Retrieved from https://app.biorender.com/biorender-templates.

2.3.5 Dysregulation of the Ubiquitin-Proteasome System in Cancer

Given the importance of ubiquitination in cellular homeostasis (Figure 7), it is not unexpected that its dysregulation is linked to a variety of illnesses, including cancer (Figure 10). Many E3 ubiquitin ligases are commonly dysregulated in malignancies through epigenetic and genetic pathways, or as a result of altered post-translational modifications in response to internal and extrinsic signals. Some E3s are encoded by genes that have been linked to familial cancer risk, such as the Von Hippel-Lindau (VHL) tumor suppressor gene in renal cell carcinoma (Gossage, Eisen, and Maher,

2015) and the BRCA1 gene in breast and ovarian malignancies (Savage and Harkin, 2015). Furthermore, large-scale genome analyses have found several E3s that have been changed by recurrent mutations or copy number changes in many cancer types. E3 dysregulation is also produced by post-transcriptional and post-translational changes such as phosphorylation, ubiquitination, or protein-protein interactions, according to research (Senft et al., 2018). Genetic and epigenetic changes in deubiquitinating enzymes, as well as genetic changes in the substrates themselves, cause further dysregulation of the system. The frequent genetic changes of the ubiquitination sites on Myc are one example of this (Welcker et al., 2004). Ubiquitination is a dynamic and reversible process that responds to stressors such as DNA damage and hypoxic, oxidative, and metabolic conditions, all of which cancer cells face. Therefore, a better understanding of how dysregulation of the ubiquitination process affects malignant transformation, tumor suppression, and therapy resistance is critical for better cancer patient outcomes.

Dysregulated cell-cycle control is a fundamental aspect of cancer cells since their proliferation proceeds essentially unchecked. In cancer, signals that regulate cell cycle entrance, progression, and arrest are typically dysregulated. As a result, DNA replication, DNA repair, and chromosomal segregation are disrupted, which can result in genomic instability (Senft et al., 2018). Therefore, dysregulation of E3s that induce proteasomal degradation of proteins involved in these processes, such as cyclins, CDK inhibitors and DNA damage repair machinery proteins, promote cancer initiation and progression. In fact, the dysregulation of the SCF (S-phase kinase-associated protein1

- Cullin 1 - F-box protein) and APC/C (anaphase-promoting complex, often known as the cyclosome) ubiquitin ligase E3 enzymes are the most studied examples (Figure 10).

APC/C has two co-activators CDC20 (cell division cycle 20) or CDH1 (CDC20-like protein 1). Even though APC/C itself is not frequently mutated in cancers, there is increasing evidence pointing at the tumor suppressor role for CDH1 and an oncogenic role for CDC20. CDH1 has been shown to be a haploinsufficient tumor suppressor since $Cdh1^{+/-}$ mice showed increased susceptibility to spontaneous epithelial tumors in various organs compared to wild type mice (Garcia-Higuera *et al.,* 2008). Furthermore, CDH1 knockdown in human bone osteosarcoma cell lines causes an accumulation of cyclin A and cyclin B, an early entry into S-phase, and a rise in DNA double strand breaks during mitosis due to the presence of replication intermediates (Greil et al., 2016). On the other hand, it was shown that CDC20's residual activity enhances evasion of anitmitotic drug-induced apoptosis and led to the discovery that CDC20 has carcinogenic potential (Brito and Rieder, 2009). In a two-stage skin cancer mouse model, localized deletion of Cdc20 causes significant metaphase arrest and apoptosis. $Cdc20^{-/-}$ MEFs transformed with oncogenic RAS^{G12V} and early region 1A (E1A) of human adenovirus type 5 also exhibit this phenotype (Manchado et al., 2010). These discoveries prompted the creation of APC/C-CDC20 inhibitors, which bind to APC/C and stop CDC20 or CDH1 activation (Zeng et al., 2010).

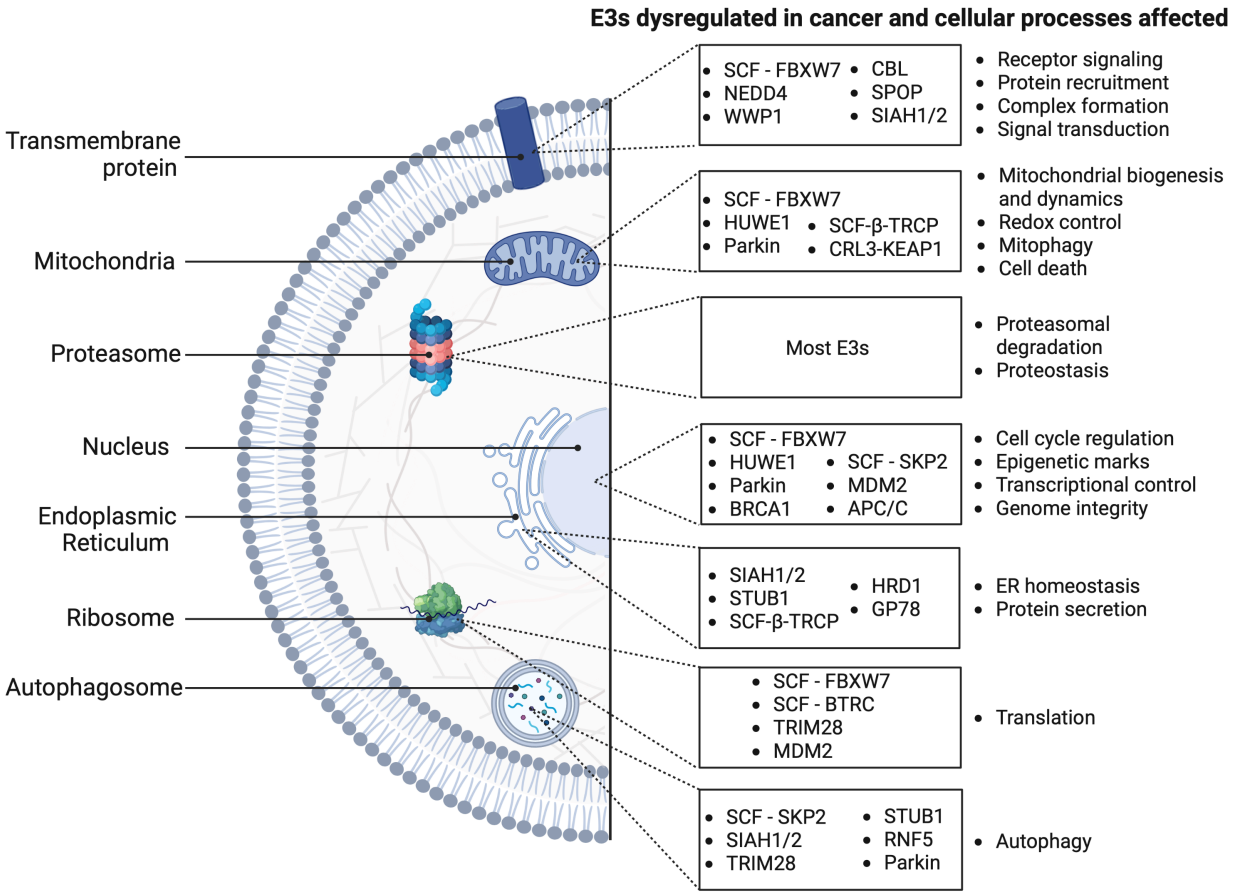


Figure 10: Cellular processes affected by dysregulated ubiquitin ligases (E3s) in cancer. As E3s ubiquitinate a diverse set of substrates, E3 loss- or gain-of-function affects multiple cellular processes simultaneously. These are representative E3s, frequently dysregulated in cancers and their downstream effects in different cellular components. Created with BioRender.com (2022).

Furthermore, F-box proteins SKP2, β-TRCP and FBXW7, which are the substrate recognition subunits of SCF E3 complexes, are frequently dysregulated in cancer, impairing its activity (Figure 10). For example, the SCF-SKP2 complex has been shown to be oncogenic since it regulates a number of CDK inhibitors, one of which is the well known tumor suppressor $p27^{KIP1}$ (Loda *et al.,* 1997; Senft *et al.,* 2020). In fact, Skp2 knockout effectively prevents prostate cancer development in a conditional Pten-deficient and Trp53-deficient mouse model by inducing senescence

through activation of $p27^{KIP1}$, $p21^{CIP1}$ and transcription factor ATF4, while Skp2 overexpression in the mouse prostate induces hyperplasia, dysplasia, and low-grade carcinoma (Shim et al., 2003; Lin et al., 2010). Additionally, SCF - β-TRCP plays two distinct roles in cell cycle checkpoint regulation: they mediate cell cycle arrest through the degradation of CDC25A (Busino et al., 2003), and they relieve arrest through the degradation of WEE1, clasping, eukaryotic elongation factor 2 kinase (eEF2K), and Fanconi anemia group M protein (FANCM) (Watanabe *et al.,* 2004; Peschiaroli *et al.,* 2006; Kruiswijk *et al.,* 2012; Kee, Kim and D'Andrea, 2009). Additionally, studies have demonstrated that SCF - β-TRCP induces cell cycle arrest by targeting for degradation CKI-phosphorylated MDM2, which stabilizes p53 (Inuzuka et al., 2010).

Out of all the E3s, MDM2 and BRCA1 lead to cancer development by regulating the DNA damage response and cell cycle checkpoints (Wade et al., 2013; Savage and Harkin, 2015). In summary, elevated levels of MDM2 is seen in many cancers since it promotes carcinogenesis primarily by targeting p53 for degradation (Wade et al., 2013). Contrarily, BRCA1 mediates monoubiquitination or non-degradative polyubiquitination of its substrates, which include histones, CtBP-interacting protein, estrogen receptor- (ER), RNA-polymerase II (RNAPII), and transcription initiation factor IIE (TFIIE), by forming a heterodimer with BRCA1-associated RING domain protein 1 (BARD1) (Savage and Harkin, 2015). Therefore, abnormal BRCA1-BRD1 activity results in errors in homologous recombination and cell cycle control, which affect genome stability.

E3s have also been implicated in signal transduction in cancer since they have the ability to regulate major growth-promoting pathways, including the MAPK or PI3K-AKT-mTOR pathways, which ar currently targeted by anticancer therapies. The E3

NEDD4 degrades RAS in a negative feedback loop where RAS activity increases levels of NEDD4, which in turn reduces the activity of wild type RAS but not oncogenic RAS (Zeng et al., 2014). In addition, NEDD4 is a known negative regulator of PTEN, therefore, NEDD4 over expression causes PTEN to degrade more rapidly, accelerating carcinogenesis of RAS-driven tumors. (Zeng et al., 2014). The interaction between RAS and NEDD4 exemplifies how a cell's genetic environment or signaling state can affect how aberrant ubiquitylation manifests itself. While NEDD4 acts as a tumor suppressor in healthy cells, it acts as an oncogene in cells that express hyperactivated and/or mutant RAS.

Sustained activation of growth and survival pathways creates a stressful environment; as a result, cancer cells must coordinately manage metabolic processes and stress signaling pathways in order to overcome these potentially detrimental conditions.

Interestingly, as seen in Figure 10, one of the most frequently dysegulated E3s is SCF-FBXW7 (F-box/WD repeat containing protein 7). FBXW7 mediate cyclin E degradation, therefore, dysregulated SCF-FBXW7 function promotes sustained proliferation and genomic instability (Grim et al., 2012). In a mouse model, intestinal FBXW7 and Trp53 co-deletion leads to advanced adenocarcinomas with high levels of cyclin E expression and a chromosomal instability phenotype (Grim et al., 2012). FBXW7 is a p53-dependent haploinsufficient tumor suppressor; thus, p53 expression can reverse the effects of FBXW7 substrate accumulation caused by heterozygous FBXW7 inactivation (Grim et al., 2012; Rajagopalan et al., 2004; Mao et al., 2004). FBXW7 has also been shown to play a direct role in DNA double-stranded break repair

(DSB). SCF-FBXW7 is phosphorylated and recruited to DSB sites as a result of ATM activation, a crucial mediator of the DNA damage response. Then, the X-ray repair cross-complementing protein 4 (XRCC4), a repair protein involved in non homologous end joining, is polyubiquitylated. NHEJ repair is increased by XRCC4 that has been K63-ubiquitinated because it is more likely to connect with the KU70 (also known as XRCC6) and KU80 (also known as XRCC5) complexes (Zhang et al., 2016). Additionally, research has demonstrated that SCF-FBXW7 controls signal transduction in cancer by ubiquitinating and encouraging the breakdown of the mTOR complex 1. (mTORC1). The relevance of FBXW7-mediated mTOR stabilization in carcinogenesis was demonstrated by the finding that deletion of a single copy of FBXW7 is mutually exclusive with loss of PTEN, a known negative regulator of mTOR, in breast cancer cell lines. As a result, FBXW7 depletion can accelerate tumor growth by mTOR accumulation, which then induces stimulation of anabolic processes.

Additionally, E3s have been linked to transcriptional regulation via controlling the abundance and activity of transcriptional activators, their ability to bind to genes and form transcriptional complexes, and the structure of chromatin (Geng *et al.,* 2012). One important case in cancer progression is the regulation of the transcription factor MYC by multiple E3 complexes, including SCF-FBXW7. Dysreguralion of the SCF-FBXW7-MYC in in vivo models of chronic myeloid leukemia causes accumulation of MYC which drives leukemia initiating cells out of quiescent state, promoting tumorigenesis (Reavie *et al.,* 2013)).

2.3.6 Ubiquitin ligases as therapeutic targets

The growing recognition and understanding of the critical roles that E3s play, and how their dysfunction can alter fundamental cellular processes, has inspired the development of targeted therapeutics against E3s. Small molecules or peptides are potential inhibitors of oncogenic E3s by repressing its expression, altering its subcellular location, preventing substrate contact, preventing assembly into multisubunit complexes, preventing homodimerization or heterodimerization, and preventing the catalytic domain from functioning properly (Senft et al., 2018).Structure-based design in conjunction with cutting-edge small-molecule screening technology is one of the current methods used for the development of E3 inhibitors, which has lead to inhibitors against APC/C (Sackton *et al.,* 2014), MDM2 (Zhang *et al.,* 2015) and SKP2 (Chan *et al.,* 2013)., among a few others. However, targeting protein-protein interactions are particularly challenging in the drug development field.

Targeting tumor suppressors in cancer therapy is still challenging in general. Reactivating an E3 that has been suppressed, investigating genetic vulnerabilities using the theory of synthetic lethality (like using PARP inhibitors in BRCA1- or BRCA2-deficient tumors; Bryant et al., 2005), or inhibiting downstream oncogenic substrates are some methods for targeting tumor suppressor E3s. For example, targeting MCL1 in malignancies using SCF-FBXW7 inactivation or a more general strategy using proteolysis-targeting chimaera (PROTAC) technology, which uses bifunctional molecules to instruct other E3s to degrade oncogenic substrates, are two ways to carry out the latter (Skaar, Pagan and Pagano, 2014).

Although targeting E3s as a novel therapeutic has great promise, the field is proceeding with caution. Because E3s can act as tumor suppressors or promoters in a substrate-dependent and context-dependent manner, targeting them requires a thorough understanding of their activity in the specific tissue and/or tumor context. Furthermore, post-translational modifications can transform an E3 from a tumor suppressor to a tumor promoter, emphasizing the complexities of E3 activity and regulation that can affect targeting. Novel therapeutics should specifically disrupt E3 interactions with substrates important in cancer biology. Therefore, a better understanding of the E3s structure, post-translational modifications, and their role in tumor progression will undoubtedly guide the next generation of novel biological and small molecule drugs for this group of proteins.

PART 3: F-box WD-repeat Containing Protein 7 (FBXW7)

3.1 Introduction

The SCF E3 is composed of four subunits: RBX1 which contains a small, zinc-binding RING finger domain where the E-Ub conjugate binds; Cullin (CUL1) which forms the major structural scaffold of the complex; SKP1 which is an adaptor protein essential for the recognition of F-box proteins; and F-box protein (FBP) which contributes to substrate specificity by first aggregating to target proteins independent of the complex. F-box proteins are an extensive family of eukaryotic proteins characterized by a carboy-terminal domain that interacts with substrates, and a 42-48 amino acid F-box motif (so named because Cyclin F was one of the first substrates identified) which binds to SKP1 (Winston *et al.,* 1999). By 1998, only four mammalian F-box proteins (Cyclin F, Skp2, β-TRCP and NFB42) were identified (Bai *et al.,* 1996; Erhardt *et al.,* 1998). Then, in 1999, two studies identified a family of 25 novel mammalian F-box proteins (Winston *et al.,* 1999; Cenciarelli *et al.,* 1999). The authors discovered 26 human F-box proteins, 25 of which were novel, by scanning DNA databases and using SKP1 as bait in a yeast two-hybrid screen. These proteins were grouped based on their domains: some had WD-40 domains or leucine-rich repeats, others had leucine-zippers, ring fingers, helix-loop-helix domains, proline-rich motifs and Src homology (SH2) domains; others did not fall in any of these groups. The F-box proteins with WD-40 domains are designated as Fbws, those with leucine-rich repeats as Fbls, and the rest as Fbxs (Winston et al., 1999; Cenciarelli et al., 1999). Nowadays, there are ~70 F-box proteins identified in humans. Among the Fbws identified was the

F-box WD-repeat Containing Protein 7, also known as FBXW7, FBXW7, Sel10, hCDC4, or hAgo.

3.2 Molecular characteristics of FBXW7

Human FBXW7 stretches over 200kb on chromosome 4 and encodes three different transcripts (FBXW7α, β and γ) by alternative splicing (Figure 11A) (Spruck *et al.,* 2002). Each transcript has ten shared exons coupled to an isoform-specific first exon, resulting in three protein isoforms that differ solely in their N-termini (Figure 11). Three functional domains are found in the common region of all isoforms: a stretch with eight-WD-40 repeats, an F-box domain that interacts with SKP1 of the SCF complex, and a dimerization domain (DD). (Figure 11C, D) (Sprunk *et al.,* 2002). The WD40 repeat stretch makes multiple contacts with the substrate. Crystallographic studies of FBXW7 (and Cdc4) have revealed that the WD40 repeats form an eight-bladed barrel-shaped -propeller structure with defined phospho-degron binding pockets (Orlicky *et al.,* 2003; Hao *et al.,* 2007). The phosphorylated substrates are centrally contacted by three highly conserved arginine residues in repeats 3 and 4, and other residues in all WD40 repeats aid in binding the substrate (Orlicky et al., 2003; Hao et al., 2007). The DD domain, which is located just before the F-box, facilitates FBXW7 dimerization, which is a feature shared by F-box proteins (Hao *et al.,* 2007). FBXW7 has been shown to form homodimers through the DD, with each monomer recruiting its own E2-Ub conjugate, which causes the distances between each FBXW7 substrate-binding domain and the E2 catalytic sites to vary. This spatial heterogeneity may enable a greater variety of lysine acceptors for ubiquitin conjugation, both in the substrate and the expanding polyubiquitin chain, improving the efficiency of substrate breakdown

(Hao et al., 2007). Because all isoforms share these three main functional domains, in principle, they are functionally identical. However, the distinct N-terminal domain of each isoforms determines subcellular localization (Kimura *et al.,* 2003). Cis-acting signals in the isoforms-specific first exon direct FBXW7α to the nucleoplasm, FBXW7 β to the cytoplasmic membranes and FBXW7γ to the nucleolus (Figure 11C). Interestingly, FBXW7γ localization signals are not very well understood since it resides within the shared FBXW7 exons (Welcker *et al.,* 2004).

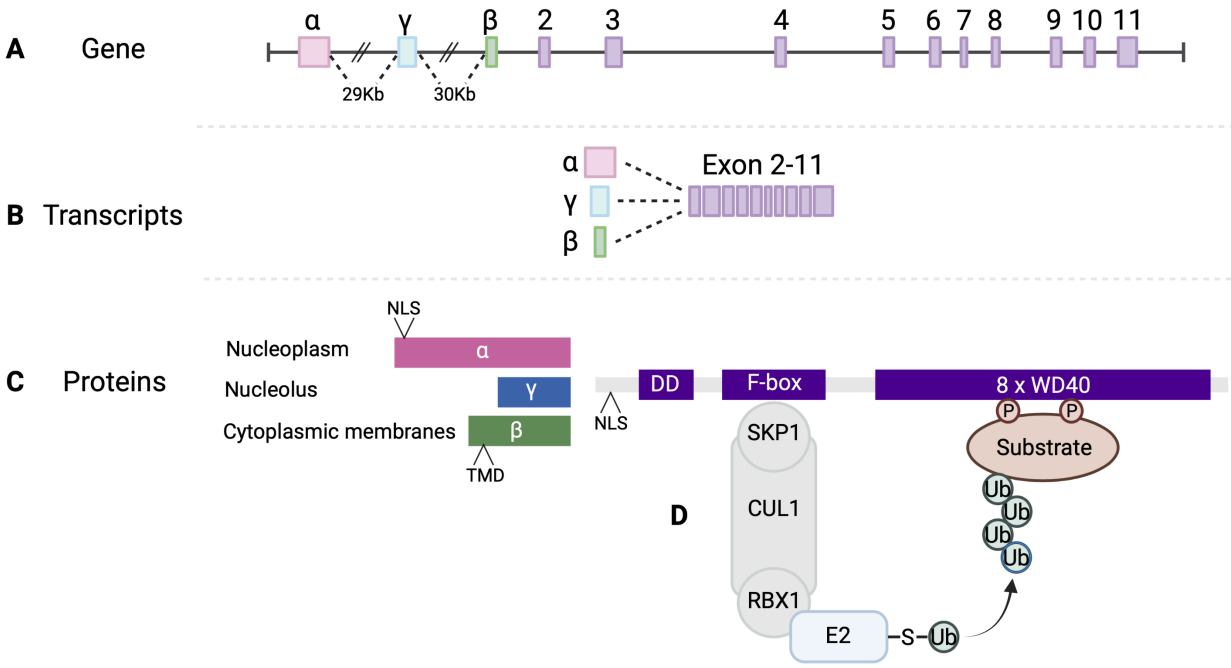


Figure 11: The organization of the FBXW7 gene and its protein isoforms. (A) Representative organization of the human FBXW7 genomic locus. Alternative splicing of the first exon leads to distinct FBXW7 transcripts **(B)** and proteins **(C)**. **(D)** Interaction between the FBXW7 functional domains and the rest of the SCF E3 complex and the substrate to be polyubiquitinated. NLS, nuclear localization; TMD, transmembrane domain. Adapted from Welcker and Clurman, 2008, and Yumimoto and Nakayama, 2020. Created with BioRender.com (2022).

Each isoform is differentially regulated, but the physiological importance of isoform-specific transcriptional regulation is still unknown. For instance, in the majority, if not all, of human cell lines and primary cells, FBXW7α mRNA is expressed at far higher levels than either FBXW7β or FB FBXW7γ XW7 (Sprunk et al., 2002). In adult mouse tissues, FBXW7α is widely expressed, however it is discovered at high levels in the brain and its expression is elevated in muscle (Matsumoto et al., 2006). Little is known about the signals that control the transcription of FBXW7, with the exception of FBXW7β, which is activated by the tumor suppressor p53. However, the majority of FBXW7's known actions seem to be attributed to the alpha isoform, with the biological significance of the betta and gamma isoforms appearing to be minimal.

3.2.1 Transcriptional regulation

It is yet unclear how FBXW7 is regulated during transcription. The chromatin immunoprecipitation database (ChIP) shows that the transcription factors encoded by the SPI1, CREB1, and RELA genes bind preferentially to the FBXW7 promoter region, in addition to fundamental elements of the transcriptional machinery like EP300, BRD4, and MED1 (Oki et al., 2018). But further research is needed to understand the significance of these connections. It has been demonstrated that the transcriptional regulator C/EBP δ directly inhibits FBXW7α transcription in breast cancer cells, leading to increased hypoxia and inflammatory signaling as a result of the accumulation of mTOR (Balamurugan et al., 2010). P53, which is triggered by genotoxic stress such as that brought on by ultraviolet irradiation, directly increases the transcription of the FBXW7β gene (Kimura et al., 2003). Additionally, HES5 directly downregulates FBXW7β

transcription (Sanxho et al., 2013). To date, no direct transcriptional regulation of FBXW7γ abundance has been identified.

3.3 FBXW7 substrates

The majority of SCF-FBXW7 substrates contain the Cdc4 phosphodegron (CPD) sequence, a conserved phosphorylation motif that typically includes threonine or serine at position 0, proline at positions +1 and +2, threonine, serine, glutamate, or aspartate at position 4, and a hydrophobic amino acid at positions 5, 3, 2, and 1 (Figure 12). (Hao et al., 2007). The WD40-repeat domains of FBXW7 recognize the substrate when serine or threonine at the 0 and +4 positions of the CPD are both phosphorylated, leading to ubiquitination and proteasomal degradation. It's interesting to note that only ~13% of the known FBXW7 substrates process a CPD with this ideal sequence. The more dissimilar the CPD is from the optimal sequence, the weaker the affinity between FBXW7 and the substrate (Hao et al., 2007). For those substrates with weak affinity, FBXW7 dimerization is essential for stable polyubiquitination of the substrate (Welcker et al., 2013).

Many of the known FBXW7 targets are proto-oncoproteins such as cyclin E, c-Myc, Mcl-1, mTOR, Jun, Notch, and AURKA, which support the role of FBXW7 as a tumor suppressor (Figure 12) (Supplementary Table 2) (Yeh *et al.,* 2018). For instance, dysregulation of the proto-oncogene c-Myc has been linked to the emergence of numerous human malignancies. According to reports, FBXW7 ubiquitinates c-Myc in both the nucleoplasm and the nucleolus, causing it to degrade and preventing it from promoting the proliferation of cancer cells (Davis et al., 2014).

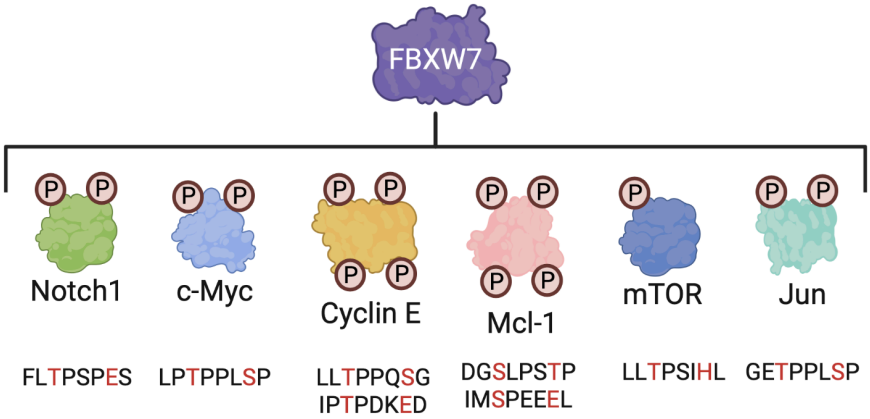


Figure 12: FBXW7 substrates and their conserved CDC4 phosphodegrons. The amino acid sequence under each substrate indicates the conserved CDC4 phosphodegron, where highlighted residues refer to the "0" and "+4" phosphorylated residues.

Cyclin E

Cyclin E and cyclin-dependent kinase Cdk2 (cyclin E / CDK2) regulate cell cycle progression by licensing DNA replication at the G1-S phase transition (Grim *et al.,* 2008). A faster rate of DNA synthesis is linked to higher levels of Cyclin E, therefore, tight regulation of Cyclin E levels is needed, which is achieved by protein kinases and phosphates pathways. FBXW7 recognizes phosphorylated Cyclin E at Ser384 and Thr380, leading to Cyclin E degradation (Koepp *et al.,* 2001). Then, phosphates remove these phosphorylation events such that FBXW7 no longer recognizes Cyclin E, leading to higher levers of Cyclin E (Davis *et al.,* 2017). Therefore, FBXW7 mutations can lead to Cyclin E accumulation, which can cause chromosomal instability, aneuploidy, and cancer (Strohmaier *et al.,* 2001; Minella *et al.,* 2007; Rajagopalan *et al.,* 2004).

c-Myc

C-Myc is a master regulator of cellular gene transcription that has been shown to bind to up to 15% of the promoters in the human genome (Fernandez *et al.,* 2003). As a result, it can alter cellular survival and proliferation pathways and is frequently dysregulated in human malignancies. Skp2 and FBXW7 are two proteins of E3 complexes that have the ability to post-transcriptionally control c-Myc turnover. In fact, multiple studies have reported that loss of FBXW7 function leads to an increase in c-Myc expression and activity (Yada *et al.,* 2004; Reavie *et al.,* 2013). FBXW7-mediated degradation of c-Myc is dependent on the phosphorylation of Thr58 and Ser62 residues in c-Myc CPD (Fernandez *et al.,* 2003; Yada *et al.,* 2004). However, mutations of these residues are frequently found in cancers leading to increased c-Myc levels and accelerated tumor progression (Bahram *et al.,* 2000).

Notch1

A family of receptors known as Notch proteins ((Drosophila) Homolog 1 Translocation-Associated) are in charge of activating Notch signaling pathways, which have a variety of cellular effects, including functions in development, cellular differentiation, control of stem cells, cellular proliferation, and cell death (Louvi and Artavanis-Tsakonas, 2012). FBXW7 targets Notch1 for degradation by recognizing phosphorylation on residues Thr1851, Thr2123, Thr2125 and Ser2173 (Foltz et al., 2002). Numerous hematological and solid cancers have been shown to contain genetic abnormalities that impede Notch1 turnover (Weng *et al.,* 2004). More than 30% of

children with T-ALL have FBXW7 mutations, which result in an extended FBXW7 half-life and resistance to Notch inhibitors (Weng et al. 2004).

MCL-1

The BCL-2 family member Myeloid Cell Leukemia-1 (Mcl-1) controls apoptosis in both healthy and malignant cells (Michels *et al.,* 2004). Following MCL-1 phosphorylation at Ser159 and Thr163, FBXW7 interacts with and degrades MCL-1 (Inuzuka *et al.,* 2011). In fact, it has been demonstrated that FBXW7 mutation in squamous cell cancer increases MCL expression and fosters resistance to conventional chemotherapy (He *et al.,* 2013).

mTOR

The mammalian target of rapamycin (mTOR) is a Ser/Thr kinase that plays crucial functions in cell growth, metabolism, survival, and autophagy. FBXW7-mediated ubiquitination and degradation controls mTOR turnover (Mao et al., 2008). FBXW7 deleted or mutant cells are more vulnerable to rapamycin treatment due to elevated mTOR expression (Mao et al., 2008). This could be utilized as a biomarker to identify people who could respond more favorably to therapy with rapamamycin.

Jun

The proteins Jun-Fos and Jun-ATF2 make up the majority of Jun dimers, which are crucial for regulating cell proliferation, stress reactions, and apoptosis. FBXW7 recognizes phosphorylation events of c-Jun at Thr239 and Ser243 and targets it for

degradation (Wei et al., 2005). The importance of FBXW7 regulating Jun in carcinogenesis is seen in animals with FBXW7 knockout in the gut. At 9–10 months of age these mice develop adenomas and exhibit increased c-Jun expression (Babaei-Jadidi *et al.,* 2011).

Aurora kinase A (AURKA)

AURKA is a serine/threonine kinase that regulates the centrosome duplication checkpoint, spindle assembly checkpoint, and cytokinesis (Mao *et al.,* 2004). AURKA's half-life is increased, according to studies, when the phosphatase and tensin homolog on chromosome 10 (PTEN) is lost (Kwon *et al.,* 2012). Further research revealed that Ser245 and Ser387 phosphorylation was necessary for FBXW7-mediated proteasome degradation of AURKA and that Thr217 and Glu221 amino acids were essential for direct contact between AURKA and FBXW7 (Kwon et al., 2012). Therefore, FBXW7 and PTEN can work together to inhibit tumor growth via targeting AURKA (Kwon *et al.,* 2012).

3.4 FBXW7 mouse models

At embryonic day 10.5, FBXW7-deficient mouse embryos pass away in utero as a result of poor yolk sac and brain vascular development (Bonetti *et al.,* 2008; Durgan and Parker, 2010). It was discovered that the FBXW7 substrate NOTCH4 accumulated in mutant embryos, and that the abundance of its downstream target HEY1 was noticeably elevated in blood vessels, indicating that NOTCH signaling dysregulation is probably what causes the vascular abnormalities. Surprisingly, accumulation of Cyclin

E levels was not observed, likely due to a regulatory decrease in Cyclin E mRNA levels. The idea that FBXW7 is a tumor suppressor is supported by the fact that FBXW7$^{+/-}$ mice appear to be healthy and fertile but exhibit an increased sensitivity to radiation-induced carcinogenesis, particularly on the Trp53$^{+/-}$ or Pten$^{+/-}$ background (Bonetti *et al.,* 2008; Durgan and Parker, 2010).

Overall, the phenotypes of mice with tissue-specific FBXW7 ablation have shown that this protein has pleiotropic effects on cell division and proliferation. For instance, FBXW7 conditional deletion in the thymus caused pronounced thymic hyperplasia, which was followed by spontaneous thymic lymphoma formation linked to the buildup of c-MYC (Welcker and Clubman, 2005). Hematopoietic stem cells (HSCs) self-renewal capacity was lost in mice lacking FBXW7, and HSCs became exhausted and c-MYC and NOTCH1 accumulated in quiescent HSCs (Thompson et al., 2008). Hepatomegaly and steatohepatitis were seen in FBXW7-specific hepatocyte-deficient animals, most likely as a result of SREBP stabilization and impacts on the expression of its target genes (Onoyama et al., 2011).

To better study the complete loss of function of FBXW7, a Cre-Lox system was developed to specifically knockout FBXW7 in tissue-specific tumors. The FBXW7 flowed allele has LoxP sites flanking exons 5 and 6, which encode the F-box and WD40 domains, such that after tissue-specific Cre-recombinase expression FBXW7 expression is lost (Thompson et al., 2008).

3.4 FBXW7 dysregulation

3.4.1 Genetic dysregulation of FBXW7

FBXW7 is located within 4q32, a frequently deleted chromosomal region in cancers (Welcker and Clurman, 2008). According to the Catalogue of Somatic Mutations in Cancer (COSMIC) database, FBXW7 is the most frequently mutated F-box protein in cancers (Figure 13A) (Forbes *et al.,* 2017). FBXW7 mutations can reduce its ability to form SCF E3 complexes, or it may change its conformation leading to non-functional complexes. Across all human tumors in COSMIC, FBXW7 is mutated in 2.54% of cases (n=1,216 / n=47,844), of which 72.70% are missense mutations and 13.82% are nonsense mutations, whereas 7.89% are insertions/deletions (Yeh *et al.,* 2018). Further examination of tissues revealed that FBXW7 point mutations are more frequently seen in the endometrium (85/918: 9.26%), large intestine (346/4512: 7.73%), cervix (24/411: 5.84%), small intestine (8/143: 5.59%) and stomach (51/1182: 4.31%) (Figure 13A). Most FBXW7 mutations are found in hotspots R465 (25.41%), R505 (13.40% and R479 (9.29%), all of which are found in the WD40 substrate recognition domains (Forbes et al., 2017). Additionally, the COSMIC database shows that residues R278, R367, G423, S582 and R689 are mutated in more than 20 distinct cancer cases. It's interesting to note that various FBXW7 mutations in the WD40 domains can influence how well-defined substrates degrade. In fact, studies have shown that the point mutant FBXW7 D510E retains its capacity to degrade Cyclin E, Mcl-1, and c-Myc, but not Notch1 (Yeh et al., 2016).

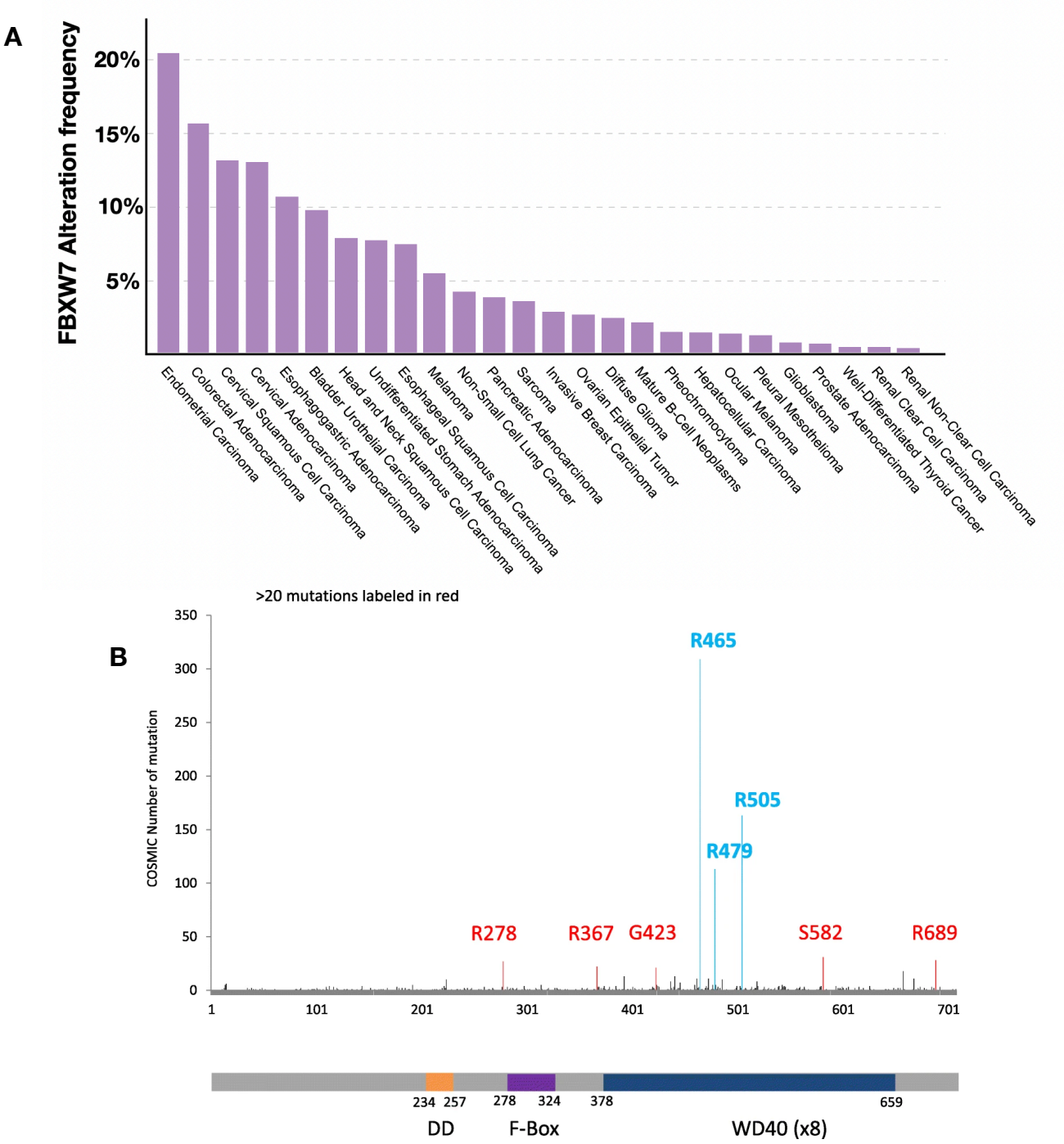


Figure 13: Genetic dysregulation of FBXW7. (A) Distribution of FBXW7 alteration frequency in human cancers. **(B)** Distribution of FBXW7 mutations across its locus. Three FBXW7 hotspot mutations are identified in blue, and FBXW7 residues with mutation numbers more than 20 are marked in red. The FBXW7 protein domains are shown in the bottom panel. (DD: dimerization domain amino acid 234–257, F-Box-like domain: amino acid 281–325 and WD40: amino acid 374–650). All data from COSMIC database. Figures adapted from Yeh *et al.,* 2016.

Due to the heterozygous mutations and deletions that are present in most cancers, FBXW7 is regarded as a haploinsufficient tumor suppressor (loss of a single allele promotes tumorigenesis). Further evidence for this theory came from a study in which it was shown that the loss of a single FBXW7 allele occurs frequently in p53-heterozygous backgrounds while mice in p53 wild type backgrounds appear to be perfectly normal (Mao et al., 2004). Recent studies on the dimerization of FBXW7 and the mutational spectrum of this protein in human cancers reveal that mutations of a single FBXW7 allele can cause dominant-negative changes in addition to loss of function. Point mutations in FBXW7 can manifest in a variety of ways. Nonsense mutations cause protein truncations, which, depending on where they occur, might result in alleles that are either inactive or possibly dominant-negative. For instance, stop codons that appear after the DD domain result in truncated FBXW7 proteins that are unable to bind substrates but may nevertheless significantly obstruct the activity of the wild-type FBXW7 protein through dimerization. Upstream of the DD domain, however, nonsense mutations are more likely to result in the production of non-functional alleles.

There are a number of mechanisms that could explain these dominant negative impacts. For instance, the formation of non-functional FBXW7 dimerizing with wild-type FBXW7, which is particularly detrimental for low affinity substrates that require dimerization for their degradation. Theoretically, this scenario could be caused by either nonsense or missense mutations anywhere downstream of the DD domain. However, hotspot mutants in cancer concentrate in the WD40 substrate recognition domains, therefore, this does not explain the whole picture. Perhaps, these full-length

hotspot mutants acquire functions that the truncated mutants lack. Hot-spot mutations, for instance, might not completely eliminate all substrate interactions, allowing these mutant proteins to bind some substrates without targeting them for degradation. By dimerizing with wild-type FBXW7 to generate inactive dimers in this scenario and by stably attaching to substrates and obstructing their accessibility to wild-type FBXW7 monomers, respectively, the hot-spot mutants could damage both the dimeric and monomeric FBXW7 function.

3.4.2 Epigenetic dysregulation of FBXW7

The FBXW7 promoter is methylated in 43% of different cancer types, which is achieved through modifications of the DNA and histone proteins (Akhoondi *et al.,* 2010). This has been associated with high-grade tumors (Akhoondi *et al.,* 2010). Interestingly, P53 mutations have been linked to hyoermethylation of the FBXW7 promoter and lower expression levels of FBXW7 (Kited et al., 2016). This may be due to p53's capacity to boost DNA methyltransferase 1 expression (DNMT1).

Moreover, histone alterations are known to regulate FBXW7 expression in addition to DNA modifications. A histone methyltransferase called Enhancer of Zeste Homolog 2 Polycomb Repressive Complex 2 (EZH2) is involved in the epigenetic silencing of many genes, including FBXW7. Three methyl groups are added by EZH2 to FBXW7's histone H3 residue (Zhao et al., 2015). This results in the FBXW7 gene's activity being silenced, which then triggers the Notch signaling pathway. It's interesting to note that FBXW7 has been found to be a substrate of EZH2, which is connected

adversely with FBXW7 expression in human PDAC samples and pancreatic cancer cells (Jin et al., 2017)

3.4.3 miRNA and lncRNA dysregulation of FBXW7

Non-coding microRNAs (miRNAs) bind to the 3' untranslated region (3' UTR) of transcript which targets the mRNA for degradation, preventing protein translation. In the case of FBXW7, it has been demonstrated that the miRNA miR-548 binds specifically to the 3'UTR of FBXW7, leading to lower levels of FBXW7 mRNA and protein (Zhang et al., 2016). Additionally, this study shows that the long non-coding RNA (lncRNA), such as lncRNA-MIF, works as a miR-548 sponge to block the effects of miR-548 (Zhang et al., 2016). Therefore, overexpression of lncRNA-MIF increases FBXW7 levels, which in turn reduces the expression of its targets such as c-Myc and c-Jun. Other miRNAs that inhibit FBXW7 are: miR-223, miR-25, miR-27, miR-32, miR-92, miR-155-3p, miR-182, and miR-503 (Yeh et al., 2016). In several of these instances, there was little or no association between a single miRNA and the expression of the FBXW7 gene in patient samples, suggesting that additional miRNAs or other regulatory mechanisms are needed to control the expression of FBXW7.

3.4.4 Post-translational dysregulation of FBXW7

Studies have shown that FBXW7 can be regulated post-translationally via auto-ubiquitination. For instance, it has been shown that the enzyme Pin1 (Peptidyl-prolyl cis-trans isomerase NIMA-interacting 1) negatively controls the FBXW7 protein by inducing conformational changes of FBXW7 that reduce its dimerization and promotes

self-ubiquitination and degradation (Min et al., 2012). This is achieved because Pin1 physically interacts with FBXW7 and binds to its Thr205-Pro are in a phosphorylation-dependent way, causing conformational shifts (Min et al., 2012). Additionally, FBXW7 can be phosphorylated at Thr205 by the extracellular signal-regulated kinase (ERK), which causes FBXW7 to be ubiquitinated and degraded by the proteasome (Ji et al., 2015). The FBXW7 mutation Thr205A provided resistance to ERK-mediated phosphorylation and decreased pancreatic cancer cell proliferation and tumorigenesis, illuminating the relevance of ERK-mediated phosphorylation of FBXW7 in PDAC (Ji et al., 2015).

Aspects of the stability of FBXW7 can also be managed by SCF ubiquitin ligase complex members. Cullin1, an adapter for FBXW7 in the SCF ubiquitin ligase complex, is controlled by CNS6's neddylation, and CNS6 encourages FBXW7's auto-ubiquitination and destruction (Chen et al., 2014). In addition, loss of one of the components of the SCF complex, known as Glomulin (Glmn), decreases FBXW7 expression by promoting Rbx1-mediated FBXW7 ubiquitination (Iron et al., 2012).

There are more than 300 distinct interacting partners for FBXW7 in the BioGrID interactions database (Chatr-Aryamontri et al., 2017). Many of which have been shown to downregulate FBXW7 levels by promoting its proteasomal degradation, as it is the case with the family with sequence similarity 83, member D (FAM83D). FAM83D is frequently dysregulated in both breast and colon cancer, and breast cancer has increased expression of FBXW7 substrates, such as c-Myc, mTOR, and c-Jun, as a result of FAM83D-mediated FBXW7 down-regulation (Wang et al., 2013).

3.5 FBXW7 and the Hallmarks of Cancer

So far, we have covered how dysregulation of FBXW7 can lead to diseases, including cancer, in a substrate-dependent manner. Here is a summary of how FBXW7 impacts the Hallmarks of Cancer, such that when dysregulated, all these pathways are altered, promoting tumorigenesis (Figure 14).

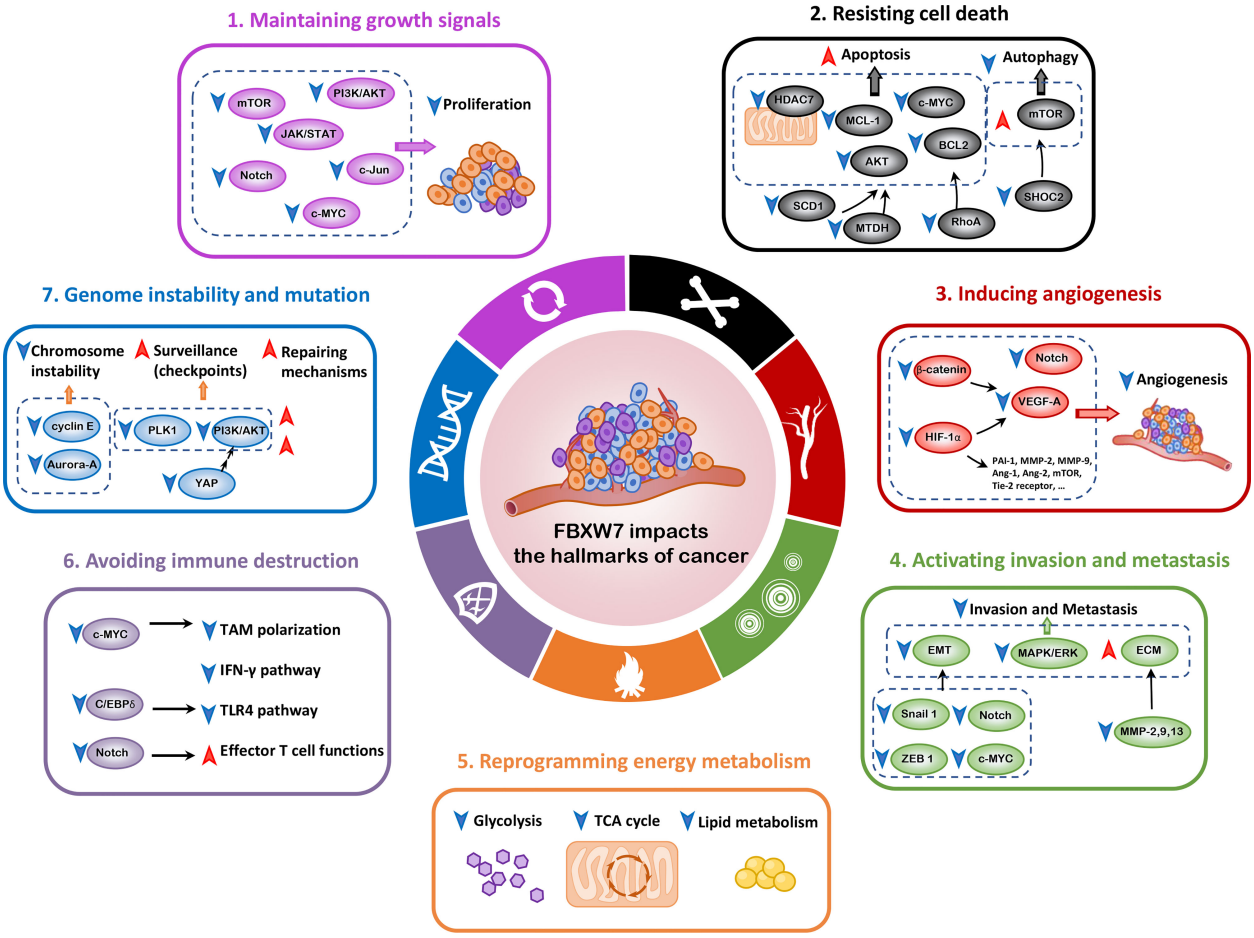


Figure 14: Role of FBXW7 in the Hallmarks of Cancer. Impact of FBXW7 in seven hallmarks of cancer. Figure taken from Chen *et al.* (2022).

1. Maintaining Growth Signals: Through the ubiquitination and degradation of multiple important signal molecules, including c-MYC, c-Jun, phosphatidylinositol 3-kinase (PI3K)/AKT, mTOR, Notch, as well as the JAK/STAT signaling cascade, FBXW7

reshapes the proliferative niches of tumor cells. Therefore, loss of FBXW7 leads to accumulation of these substrates in uncontrolled proliferation of cancer cells.

2. Resisting Cell Death: FBXW7 regulated apoptosis cascades through ubiquitination and degradation of MCL1. MCL1 is a member of BCL2 protein family, and functions as a crucial anti-apoptotic regulator. Therefore, dysregulation of FBXW7 leads to MCL1 accumulation, resisting cell death, and leading to chemotherapeutic resistance. Additionally, FBXW7 controls mTOR directly by ubiquitinating and degrading it. In response to growth hormones and an abundance of nutrients, mTOR is a crucial inhibitory regulator of autophagy. Therefore, accumulation of mTOR promotes auto-Nagy, which is protective to cancer cells.

3. Inducing angiogenesis: Angiogenesis is crucial in providing solid tumors with the oxygen and nutrients they need to meet their constantly increasing metabolic needs. The GSK-3β/FBXW7 arm was found to affect angiogenesis and metastasis by ubiquitinating and degrading the hypoxia-inducible factor HIF-1 (Lv et al., 2016). This heterodimeric transcription factor induces the expression of factors that promote angiogenesis such as VEGF, VEGF-R1, VEGF-R2, plasminogen activator inhibitor-1 (PAI-1), matrix metalloproteinase-2 (MMP-2) and MMP-9, Angioprotein-1 (Ang-1) and Ang-2 (Lv et al., 2016). Therefore, there is an increase in angiogenic activities that are advantageous to cancer cells by downregulating FBXW7 and increasing HIF-1-induced VEGF-A production. Interestingly, FBXW7 can deactivate the β-catenin pathway to affect how much VEGF-A is expressed, thereby decreasing ovarian angiogenesis.

4. Activating invasion and metastasis: The process of epithelial cells acquiring mesenchymal characteristics, known as epithelial-mesenchymal transition (EMT), is

essential for the development of malignant traits such migration, invasion, stemness, and resistance to treatment (Thiery, 2002). FBXW7 inhibits EMT upstream transcription factors like Snail 1 and zinc-finger E-box-binding homeobox 1 (ZEB1), inhibiting EMT processes (Li et al., 2016). As a result, downregulation of FBXW7 reverses its inhibitory function and encourages the spread of metastatic processes.

5. Reprogramming Energy Metabolism: Cancer cells exhibit accelerated glucose consumption, rapid ATP generation, and the conversion of glycolytic pyruvate to lactate, collectively known as "the Warburg effect," in the presence of hypoxia. Through the ubiquitin-dependent degradation of essential metabolic components as mTORC1, SREBP, HIF-1, c-MYC, and PGC-1, FBXW7 controls the rewiring of the metabolic network. Because of this, FBXW7 promotes metabolic reprogramming, which makes it easier for cancer cells to meet their early-stage nutritional needs for vital energy, anabolic processes, and redox functions.

6. Avoiding immune detection: The majority of tumor-associated macrophages are anti-inflammatory and pro-tumorigenic M2 macrophages. FBXW7 can slow cancer progression by preventing immunosuppressive niche formation and immune evasion. FBXW7 inhibits M2 polarization through regulating c-Myc degradation, as c-Myc has been shown to interact directly with the promoters of M2 macrophage-associated genes (Zhong et al., 2020). Indeed, animals with FBXW7-deficient macrophages had more aggressive tumor kinetics (Zhong et al., 2020).

7. Genome instability and mutation: Cyclin E, an FBXW7 substrate, promotes the G1/S phase transition. Cyclin E is frequently overexpressed in many human malignancies, which is connected to chromosome instability and cell cycle disruption.

Because of this, FBXW7 mutations cause an abnormal buildup of cyclin E, which results in improper chromosomal congression during metaphase and subsequent chromosome transmission. These genetic changes favor subclones of cells with superior mutant genotype to advance and take the lead within the local environment, promoting the acquisition of other cancer cell characteristics.

3.6 FBXW7 Dysregulation in Pancreatic Cancer

According to the COSMIC database, FBXW7 is mutated in ~3.8% of pancreatic cancer patients (Figure 13A), and in fact, FBXW7 ranks among the top 50 mutated genes in pancreatic cancer according to the TCGA Genome Data Analysis Center (Figure 4). In addition, looking at the Human Protein Atlas database, those pancreatic cancer patients with low FBXW7 expression have a poor probability of survival compared to patients with high FBXW7 expression (Figure 15). Collectively, this supports that FBXW7 behaves as a tumor suppressor in PDAC.

Several studies have looked further at the role of FBXW7 in pancreatic cancer progression. For instance, Calhoun *et al.* (2003) investigated the relationship of two potential mutational targets, *BRAF* and *FBXW7*, and their association with distinct subsets of pancreas carcinomas. Using pancreatic cancer microarrays and immunohistochemistry, the authors found that 6% of pancreatic adenocarcinomas overexpress cyclin E, which was partially explained by the presence of mutations in FBXW7 at the exons 8 and 9.

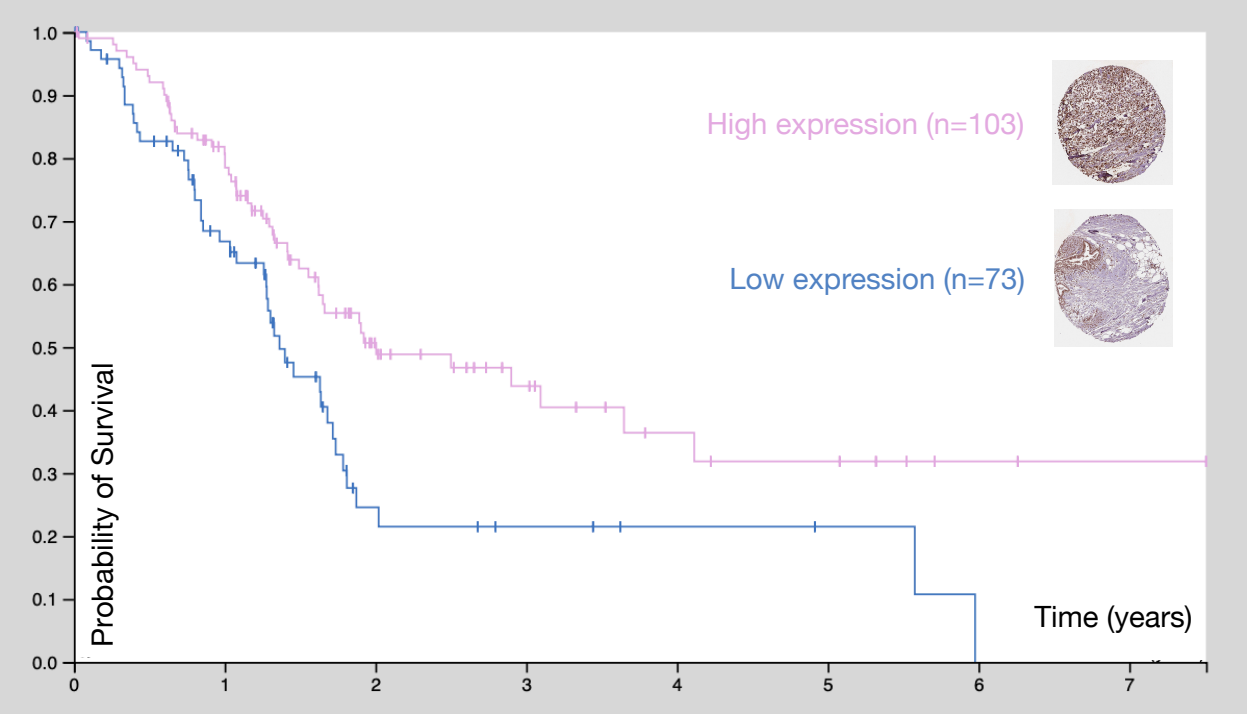


Figure 15: Probability of survival of pancreatic cancer patients based on FBXW7 expression levels. Representative histology images stained with FBXW7 antibodies validate the high vs. low expression of FBXW7. Adapted from the Human Protein Atlas. Data available from https://v15.proteinatlas.org/ENSG00000109670-FBXW7/tissue

According to Ishii et al. (2017), FBXW7 is a crucial regulator of the PDAC tumor's ability to become malignant, and its substrate MCL-1 regulates treatment resistance. In 122 pancreatic cancer tissues, the authors used immunohistochemistry to assess FBXW7 expression. Reduced FBXW7 expression was an independent predictor of poor prognosis and was substantially correlated with advanced venous invasion, increased MCL-1 expression, and elevated Ki-67 expression. Reduced FBXW7 expression was also substantially related with a poor prognosis among patients who received gemcitabine therapy following surgery. Additionally, in vitro knockdown of FBXW7 led to enhanced cell proliferation, migration, and invasion capacities as well as induced gemcitabine and nab-paclitaxel chemoresistance in pancreatic cancer cells, further

validating their findings. Moreover, the authors found that the enhanced chemo-resistance seen in the FBXW7 knockdown pancreatic cancer cells was eliminated by MCL-1 inhibition. Therefore, the FBXW7/MCL-1 axis may be a promising therapeutic approach to treat pancreatic cancer that is refractory to chemotherapy.

Gao et al. (2014) explored the mechanism of action by which nuclear export inhibitors prevent the proliferation of pancreatic cancer cells *in vitro* and slow down tumor growth. The researchers discovered that the chemical triggers G2-M cell cycle arrest and apoptosis in low nano molar ranges (IC50s~150 nM), inhibits PDAC cell growth and migration, and prevents tumor invasion, which was associated with nuclear retention of FBXW7. The proposed mechanism is that FBXW7 retention in the nucleus leading to decreased abundance of tumor promising markers such as Notch1, c-Myc, Cyclin-D1, Hes1 and VEGF.

Additionally, Jiang *et al.* (2016) described that FBXW7 is downregulated upon pancreatic cancer development. The authors showed that FBXW7 plays a tumor suppressor role in PDAC. The authors found that high levels of FBXW7 hindered pancreatic cancer cell growth and invasion, by the degradation of β-catenin leading to reduced activation of the WNT/β-catenin signaling pathway. The research demonstrated that the FBXW7/β-catenin axis also controls c-Myc transcription in pancreatic cancer cells, a well-known FBXW7 target. These findings suggest that the abnormal activation of Wnt signaling typically observed in PDAC may be caused by FBXW7 inactivation in pancreatic cancer tissues.

Additionally, FBXW7 has been connected to the control of ferroptosis and apoptosis in pancreatic cancer cells (Ye et al., 2021). This research demonstrated that

FBXW7 increased ferroptosis, a non-apoptotic form of cell death, and controlled lipid peroxidation. They discovered that FBXW7 blocked nuclear receptor subfamily 4 group A member 1 (NR4A1) in order to suppress the production of stearoyl-CoA desaturase (SCD1). According to reports, SCD1 inhibits ferroptosis as well as apoptosis, which is in line with the roles of FBXW7 and NR4A1, another gene that is downregulated by FBXW7. The authors also discovered that gemcitabine's cytotoxic activity was greatly potentiated by the FBXW7-NR4A1-SCD1, opening up new areas for chemotherapeutic intervention. Targeting FBXW7, for example, can get around resistance to targeted therapy.

Most of the studies that look at FBXW7 in pancreatic cancer have been mainly performed *in vitro* in 2D cell lines and xenograft models, which are not physiologically relevant. On the other hand, Zhang *et al.* (2016) looked at the contribution of FBXW7 to pancreatic tumorigenesis in mouse models of PDAC.

The authors developed a P48-Cre; LSL-$Kras^{G12D/+}$; $Fbxw7^{fl/fl}$ (KFC fl/fl) mouse model to examine any potential interactions between the Kras mutation and FBXW7 inactivation in pancreatic carcinogenesis. They discovered all KFC fl/fl mice developed PDAC lesions by 40 days, with PDAC onset occurring by 2 weeks of age. PDAC in KFC fl/fl mice was associated with chromosomal instability, the buildup of FBXW7 substrates Yes-associated protein (Yap), c-Myc, and Notch, and was preceded by faster onset of acinar-to-ductal metaplasia (ADM) and pancreatic intraepithelial neoplasia (PanIN) lesions. The authors also discovered that Yap silencing inhibited the growth promoted by FBXW7 deletion. Putting this data together, it shoes that FBXW7 is a strong tumor suppressor in $Kras^{G12D}$-driven PDAC, in part because of Yap’s action.

Additionally, it has been demonstrated that FBXW7 controls Notch signaling to control stem cell self-renewal and differentiation in several organs. Using Pdx1-Cre, Sancho et al. (2014) demonstrated that conditional FBXW7 knockout in embryonic pancreatic progenitor cells or inducible deletion of FBXW7 in ductal cells of the adult pancreas, enhances cell neogenesis through ductal-to-β cell transdifferentiation. Their research shoes that when FBXW7 was phosphorylated at Ser183 by GSK3β, it facilitates the ubiquitination and degradation of neurogenic 3 (Ngn3), a crucial regulator for the formation of an endocrine cell identity in the pancreas. Patients with diabetes, one of the greatest health issues in the world and a risk factor for PDAC, should pay particular attention to this. Therefore, further research on targeting the FBXW7/Ngn3 axis for *in vivo* neogenesis of β cells has great potential for the treatment of diabetes and subsequently lowering the probability of developing PDAC.

3.6 Potential Therapeutic Strategies against FBXW7

Tumors can be resistant due to the combination of multiple factors, including physical barriers, tumor heterogeneity, tumor burden and growth dynamics, the tumor microenvironment, and "undraggable" genetic drivers (such c-MYC and TP53). The role of FBXW7 in many of the cancer hallmarks provides important insights into the relationship between FBXW7 and therapeutic resistance, highlighting the importance of identifying novel targets for precision therapy.

The creation of FBXW7 agonists has received some attention in the past years. Leukemia and lymphoma cells undergo apoptosis when the natural substance oridonin stimulates FBW7-mediated proteasomal degradation of c-Myc (Huang et al., 2012). Additionally, a small molecule screening discovered SCF-12, which allosterically inhibits the substrate recognition function of FBP Cdc4 in yeast (Orlicky et al., 2010). SCF-12 binds to the WD40 domain of Cdc4 between two beta strands. Even though SCF-I2 did not inhibit Cdc4 activity in vivo, it shows great potential to allosterically inhibit the WD40 substrate recognition domains

The SCF-FBXW7 axis has been notably difficult to therapeutically target in the field. However, with an increased understanding of ubiquitination, and the relationship between structures/functions, we will improve the rational design of selective therapeutics and tool compounds by targeting this critical pathway. However, the field is moving with caution. FBXW7 can act as tumor suppressors or promoters in a substrate-dependent, therefore, one must understand in depth their activity in the specific tissue and/or tumor context.

Even though, the role of FBXW7 in pancreatic cancer has been studied to some extent, its role in pancreatic progression to metastasis is still a big question in the field. In Chapter 2 I will outline efforts to further understand the role of FBXW7 in metastatic progression of PDAC using genetically engineered mouse models.

Supplemental Tables

Supplemental Table 1: Mouse models of pancreatic cancer

(Adapted from Saki et al.)

Genotype	Time of expression	Phenotype	Reference
Sox9CreER;*Kas*$^{LSL\text{-}G12D}$;*Trp53*$^{flox/flox}$ *Ptf1aCreER*;*Kras*$^{LSL\text{-}G12D}$;*Trp53*$^{flox/flox}$	Inducible	PanIN, PDAC	Lee, 2019
Tg(Ela^{5}*-Kras*^{G12D}*)*	~P30	Preinvasive ductal neoplasia, acinar cell dysplasia PanIN, PDAC	Grippo, 2003
Pdx1-Cre;*LSL-Kras*^{G12D}	E8.5	PanIN, PDAC	Hingorani, 2003
Ptf1$^{Cre/+}$;*LSL-Kras*^{G12D}	E9.5	PanIn, PDAC	Hingorani, 2003
Kras^{G12Vgeo};*Elas-tTA/tetO-Cre*	Inducible	PanIN, PDAC	Guerra, 2007
Tg(CAG-lox-GFP-stop-lox-Kras^{G12V}*);Hinf1b/CreER*^{T2}	Inducible	PanIN, PDAC	Singh, 2021
Pdx1-Flp;*FSF-Kras*$^{G12D/+}$;*FSF-R26*$^{CAG\text{-}CreERT2}$	E9.5	PanIN, PDAC	Schönhuber, 2014
Pdx1-FlpO;*Frt-STOP-Frt kras*^{G12D}	E9.5	PanIN, PDAC	Wu, 2017
Pdx1-CreER^{T2};*Braf*$^{CA/+}$	Inducible	PanIN	Collisson, 2012
Tg(Pdx1-Cre)Pik3^{cap110*}	E8.5	PanIN, PDAC	Payne, 2015
Tg(Pdx1-Cre);*Pik3ca*^{H1047R}	E8.5	PanIN, PDAC	Payne, 2015
Sox9-CreER^{T2};*Pten*$^{flox/flox}$;*LSL-Kras*^{G12D}	Inducible	IPMN, PDAC	Kopp, 2018
Tg(Ela-1-myc)	~P30	Mixed acinar/ ductal adenocarcinoma	Sandgren, 1991
Pdx1-Cre;*CAG-tTA*;*TetO-Myc*	Inducible	PanIN, PDAC	Lin, 2013
Pdx1-Cre;*CAG-tTA*;*TetO-Kras*^{G12D}	Inducible	PanIN, PDAC	Rajbhandari, 2017
Pdx1CreER;*Kras*^{G12D};*Trp53*$^{fl/+}$; *Rosa*$^{confetti/YFP}$	Inducible	PanIN, PDAC	Maddipato, 2021

Tg(CAG-LSL-GNAS^{R201H}); *LSL-Kras^{G12D}*;*Ptf1$^{Cre/+}$*	E9.5	IPMN	Taki, 2016
Sox9-CreER^{T2};*LSL- Kras^{G12D}*;*Lkb1$^{flox/flox}$*	Inducible	IPMN	Collet, 2020
Pdx1-Cre; *LSL-Kras^{G12D}* ; *Smad4$^{flox/flox}$*	E8.5	IPMN, PanIN	Bardeesy, 2006
Pdx1-Cre;*LSL-Kras^{G12D}*;*Ink4a/Arf$^{flox/flox}$*;*Smad4$^{flox/flox}$*	E8.5	IPMN differentiated PDAC	Bardeesy, 2006
Pdx1-Cre; *LSL-Kras^{G12D}*; *Ink4a/Arfflox/flox*	E8.5	PanIN, poorly differentiated PDAC	Aguirre, 2003
Ptf1$^{Cre/+}$;*LSL-Kras^{G12D}*;*LSL-Trp53$^{R172H/+}$*	E9.5	IPMN, PDAC	Hingorani, 2005
Ptf1a-CreER;*LSL- Kras^{G12D}*;*Tp53$^{loxP/+}$*;*LSL-Gnas^{R201C}*	Inducible	IPMN, PDAC	Patra, 2018
LSL-Kas^{G12D};*ptf1a$^{+/cre}$*;*ATM$^{loxp/loxP}$*	E8.5	PDAC, metastasis	Misha, 2020
Ptf1$^{Cre/+}$;*LSL- Kras^{G12D}*;*rtTA3$^{lox/lox}$*;*sgRnf43*;*Tre3g- Cas9*	Inducible gene editing	PanIN, PDAC	Kimura, 2018
Ptf1Cre/+;*LSL-KrasG12D*;*Arid1aflox/flox*	E9.5	IPMN, PDAC	Wang, 2019
Ptf1Cre/+,*LSL-KrasG12D*;*Brg1flox/flox*	E9.5	IPMN, PDAC	Von Figura, 2014

Supplemental Table 2: FBXW7 substrates segregated by CPD

(Adapted from Yumimotoa and Nakayama, 2020)

Optimal CDC4 phosphodegron (CPD):

Name	Localization	CPD sequence	CPD mutation (%)	Reference
c-MYC	N	53-FELLP **T** PPL **S** -62	18.60	Yada, 2004
N-MYC	N	53-FELLP **T** PPL **S** -62	3.99	Otto, 2009
OASIS	ER to N	202-VQMPP **T** PPS **S** -211	2.45	Yumimoto, 2013
BBF2H7	ER to N	200-LHLPP **T** PPS **S** -209	0.65	Yumimoto, 2013
GATA2	N	171-FGFPP **T** PPK **E** -180	1.09	Nakajima, 2015
GATA3	N	151-FTFPP **T** PPK **D** -160	1.33	Kitagawa, 2014
NFκB2	C to N	702-LCPLP **S** PPT **S** -711	1.49	Arabi, 2012
NONO	N	423-GTLGL **T** PPT **T** -432	1.09	Alfano, 2017
DISC1	Mito, C, CS	193-PEVPP **T** PPG **S** -202	4.67	Yalla, 2017
Cyclin E1	N	390-PSGLL **T** PPQ **S** -399	2.65	Welcker, 2003
		72-CSLIP **T** PDK **E** -81	1.59	
PGC1α	N	290-GTAGL **T** PPT **T** -299	0.00	Housley, 2009
		258-LSLPL **T** PESP-267	2.20	
KLF2	N	239-ARGLL **T** PPA **S** -248	0.00	Wang, 2013
		168-PPPPD **T** PPL **S** -177	0.00	

Semiptimal CDC4 phosphodegron (CPD):

Name	Localization	CPD sequence	CPD mutation (%)	Reference
KLF5	N	298-TYFPP **S** PPS **S** -307	22.97	Liu, 2010
KLF7	N	132-AVTSL **T** PPS **S** -141	11.49	Sugiyama, 2019
KLF10	N	88-PAFCL **T** PPY **S** -97	2.70	Yu, 2018
KLF13	N	114-AAAPP **S** PAW **S** -123	1.89	Kim, 2012
SOX9	N	231-SQGPP **T** PPT **T** -240	1.90	Hong, 2016

SOX10	C to N	235-SHGPP **T** PPT **T** -244	1.06	Lv, 2015
MyRF	ER to N	133-GTLPD **S** PPD **S** -142	1.52	Nakayama, 2018
GRα	C to N	399-PDVVS **S** PPS **S** -408	0.45	Malyukova, 2013
SREBP1	ER to N	421-VEDTL **T** PPP **S** -430	0.74	Sundqvist, 2005
SREBP2	ER to N	427-NVLLM **S** PPA **S** -436	3.57	Sundqvist, 2005
C/EBPα	N	221-QPGHP **T** PPP **T** -230	1.32	Bengoechea-Alonso, 2010
C/EBPδ	N	151-AAGQP **T** PPT **S** -160	0.00	Balamurugan, 2013
BRG1	N	26-AMLGP **S** PGP **S** -35	0.28	Huang, 2018
EGLN2	N	396-VQVPV **S** QPP **T** -405	2.86	Takada, 2017
MED13	N	321-SSVTL **T** PPT **S** -330	2.64	Davis, 2013
MED13L	N	321-CGMPL **T** PPT **S** -330	0.17	Davis, 2013
NOTCH1	PM to N	2506-EHPFL **T** PSP **E** -2515	11.09	O'Neil, 2007
NOTCH2	PM to N	2411-EHPYL **T** PSP **E** -2420	0.43	Conservation with NOTCH1
NOTCH3	PM to N	2241-EHPYL **T** PSP **E** -2250	0.22	Conservation with NOTCH1
NOTCH4	PM to N	1968-PPPCL **T** PSP **E** -1977	1.26	Conservation with NOTCH1
c-JUN	N	234-EMPGE **T** PPL **S** -243	1.16	Wei, 2005
JUNB	N	250-RSRDA **T** PPV **S** -259	5.81	Perez-Benavente, 2013
TGIF1	N	359-SGLFN **T** PPP **T** -368	4.48	Bengoechea-Alonso, 2010
HSF1	N, CS	298-KEEPP **S** PPQ **S** -307	2.92	Courtis, 2015
IRF1	N	175-VEQAL **T** PAL **S** -184	0.94	Garvin, 2019
GFI1	N	89-FWRPP **S** PSA **S** -98	2.90	Kuai, 2019
SNAIL	N	102-PPSPP **S** PAP **S** -111	3.75	Xiao, 2018
REV-ERBα	N	269-FPQQL **T** PPR **S** -278	0.55	Zhao, 2016
BLM	N	166-SKSFV **T** PPQ **S** -175	0.18	Kharat, 2016
BRAF	C, N	396-TGLSA **T** PPA **S** -405	0.00	De la Cova, 2012

NCOA3	C to N	500-VAGVH **S** PMA **S** -509	0.05	Wu, 2007
Presenilin-1	PM	111-GQLIY **T** PFT **E** -120	0.00	Li, 2002
NRF1	ER to N	374-DFLLF **S** PEV **E** -383	4.03	Biswas, 2011
RICTOR	C	1690-EAVLA **T** PPKQ-1699	0.29	Koo, 2015
NGN 3	N	178-QAGSL **S** PAA **S** -187	5.50	Sancho, 2014
EZH2	N	256-LPPEC **T** PNI **D** -265	0.45	Jin, 2017
FOXM1	N	484-LEEWP **S** PAP **S** -493	1.42	Chen, 2016
FAAP20	N	108-GGHLE **S** PAR **S** -117	4.35	Wang, 2016
ZNF322A	N	386-KGLEL **S** PPHA-395	10.53	Liao, 2017
CRY2	C to N	295-VKRNS **T** PPL **S** -304	1.99	Fang, 2015
p53	C, N, ER	28-ENNVL **S** PLP **S** -37	0.22	Cui, 2020
SETD3	C to N	369-ALHFTEPPI **S** -378	0.61	Cheng, 2017
SHOC2	C to N	502-GENLL **T** HLP **E** -511	2.44	Xie, 2019
Aurora-A	CS	212-YAPLG **T** VYR **E** -221	2.02	Kwong, 2012
Cyclin E2	N	387-NGGIM **T** PPK **S** -396	1.79	Klotz, 2009
		69-CIIIE **T** PHK **E** -78	0.89	
MCL1	N, Mito	116-ADAIM **S** PEE **E** -125	2.94	Inuzuka, 2011
		154-TSTDG **S** LPS **T** -163	3.53	
DEK	N	10-GEGTP **T** QPA **S** -19	3.70	Akhoondi, 2007
		62-KVERL **T** MQV **S** -71	1.85	
CCDC6	CS	422-KFKRP **T** PPP **S** -431	2.10	Zhao, 2012
		408-GITRP **S** PRR **S** -417	2.80	
CDX2	N	55-LDSAQ **S** PGP **S** -64	4.50	Kumar, 2016
		278-VPEPL **S** PVS **S** -287	0.00	
DAB2IP	PM	134-HESLL **S** PSSA-143	0.87	Dai, 2014
		697-FTRLP **S** PTP **E** -706	2.04	
EBP2 *	NL	1-MD **T** PPL **S** -7	1.63	Welcker, 2011
LSD1 *	N	776-MAQPI **T** PGP **S** -785	1.12	Lan, 2019

c-MYB	N	567-SSVLM **T** PVS **E** -576 **		Kitagawa, 2009
Fetuin-A	S	300-LRHAF **S** PVA **S** -309 ***		Zhao, 2018

Other CDC4 phosphodegron (CPD):

Name	Localization	CPD sequence	CPD mutation (%)	Reference
NF1	N	2752-EESLL **T** PTSP-2761	0.17	Tan, 2011
TOPOIIα	N	1356-PKTKT **S** PKL **S** -1365	0.00	Chen, 2011
EYA1	N	152-LSQSQ **S** PGQ **T** -161	0.27	Sun, 2014
PLK1	N, CS	209-KTLCG **T** PNYI-218	1.21	Giraldez, 2014
PLK2	CS	238-RTICG **T** PNYL-247	0.87	Conservation with PLK1
PLK3	N, CS, Ga	218-KTICG **T** PNYV-227	1.44	Conservation with PLK1
PLK4	N, CS	169-YTLCG **T** PNYI-178	0.00	Conservation with PLK1
NDE1	CS	186-QEKPR **T** PMP **S** -195	3.17	Maskey, 2015
mTOR	Lyso, C, Mito, ER, Ga, N	626-CSRLL **T** PSIH-635	0.37	Mao, 2008
PTPN11	N	549-LADQT **S** GDQ **S** -558	0.91	Song, 2017
HIF1α	N	493-QIQDQ **T** PSP **S** -502	1.73	Cassavaugh, 2011
Aurora-B	N, CS	Not found		Teng, 2012
ZEB2	N	Not found		Li, 2019
p63	N	Not found		Galli 2010
RCAN1	N	Not found		Lee, 2012
YAP	N	Not found		Tu, 2014
NRF3	N	Not found		Kannan, 2015
ENO1	PM, C, N	Not found		Zhan, 2015
GCSFR	PM	Not found		Ocher, 2013
STAT3	C to N	Not found		Yao, 2017

RHOGDIα	C	Not found		Zhu, 2017
MTDH	ER	Not found		Chen, 2018
NDRG1	C	Not found		Gasser, 2014
XRCC4	N	Not degraded		Zhang, 2016
γ-Catenin	C to N	Not degraded		Li, 2018

* A pseudosubstrate.

** Mouse sequence; T572 is not conserved in human.

*** Mouse sequence; S305 is not conserved in human.

References

Aguirre, A. J., Bardeesy, N., Sinha, M., Lopez, L., Tuveson, D. A., Horner, J., Redston, M. S., & DePinho, R. A. (2003). Activated Kras and Ink4a/Arf deficiency cooperate to produce metastatic pancreatic ductal adenocarcinoma. *Genes & Development*, *17*(24), 3112–3126. https://doi.org/10.1101/GAD.1158703

Akhoondi, S., Lindström, L., Widschwendter, M., Corcoran, M., Bergh, J., Spruck, C., Grandér, D., & Sangfelt, O. (2010). Inactivation of FBXW7/hCDC4-β expression by promoter hypermethylation is associated with favorable prognosis in primary breast cancer. *Breast Cancer Research: BCR*, *12*(6), R105. https://doi.org/10.1186/bcr2788

Alfano, L., Caporaso, A., Altieri, A., Costa, C., Forte, I. M., Iannuzzi, C. A., Barone, D., Esposito, L., Giordano, A., & Pentimalli, F. (2018). NONO ubiquitination is mediated by FBW7 and GSK3 β via a degron lost upon chromosomal rearrangement in cancer. *Journal of Cellular Physiology*, *233*(5), 4338–4344. https://doi.org/10.1002/jcp.26269

Arabi, A., Ullah, K., Branca, R. M. M., Johansson, J., Bandarra, D., Haneklaus, M., Fu, J., Ariës, I., Nilsson, P., Den Boer, M. L., Pokrovskaja, K., Grandér, D., Xiao, G., Rocha, S., Lehtiö, J., & Sangfelt, O. (2012). Proteomic screen reveals Fbw7 as a modulator of the NF-κB pathway. *Nature Communications*, *3*, 976. https://doi.org/10.1038/ncomms1975

Babaei-Jadidi, R., Li, N., Saadeddin, A., Spencer-Dene, B., Jandke, A., Muhammad, B., Ibrahim, E. E., Muraleedharan, R., Abuzinadah, M., Davis, H., Lewis, A., Watson, S., Behrens, A., Tomlinson, I., & Nateri, A. S. (2011). FBXW7 influences murine intestinal homeostasis and cancer, targeting Notch, Jun, and DEK for degradation. *The Journal of Experimental Medicine*, *208*(2), 295–312. https://doi.org/10.1084/jem.20100830

Bahram, F., von der Lehr, N., Cetinkaya, C., & Larsson, L. G. (2000). C-Myc hot spot mutations in lymphomas result in inefficient ubiquitination and decreased proteasome-mediated turnover. *Blood*, *95*(6), 2104–2110.

Bai, C., Sen, P., Hofmann, K., Ma, L., Goebl, M., Harper, J. W., & Elledge, S. J. (1996). SKP1 connects cell cycle regulators to the ubiquitin proteolysis machinery through a novel motif, the F-box. *Cell*, *86*(2), 263–274. https://doi.org/10.1016/s0092-8674(00)80098-7

Balamurugan, K., Sharan, S., Klarmann, K. D., Zhang, Y., Coppola, V., Summers, G. H., Roger, T., Morrison, D. K., Keller, J. R., & Sterneck, E. (2013). FBXW7α attenuates inflammatory signalling by downregulating C/EBPδ and its target gene Tlr4. *Nature Communications*, *4*, 1662. https://doi.org/10.1038/ncomms2677

Balamurugan, K., Wang, J.-M., Tsai, H.-H., Sharan, S., Anver, M., Leighty, R., & Sterneck, E. (2010). The tumour suppressor C/EBPδ inhibits FBXW7 expression and promotes mammary tumour metastasis. *The EMBO Journal*, *29*(24), 4106–4117. https://doi.org/10.1038/emboj.2010.280

Bardeesy, N., Cheng, K. H., Berger, J. H., Chu, G. C., Pahler, J., Olson, P., Hezel, A. F., Horner, J., Lauwers, G. Y., Hanahan, D., & DePinho, R. A. (2006). Smad4 is dispensable for normal pancreas development yet critical in progression and tumor biology of pancreas cancer. *Genes & Development*, *20*(22), 3130–3146. https://doi.org/10.1101/GAD.1478706

Basturk, O., Hong, S. M., Wood, L. D., Adsay, N. V., Albores-Saavedra, J., Biankin, A. V., Brosens, L. A. A., Fukushima, N., Goggins, M., Hruban, R. H., Kato, Y., Klimstra, D. S., Klöppel, G., Krasinskas, A., Longnecker, D. S., Matthaei, H., Offerhaus, G. J. A., Shimizu, M., Takaori, K., … Furukawa, T. (2015). A Revised Classification System and Recommendations From the Baltimore Consensus Meeting for Neoplastic Precursor

Lesions in the Pancreas. *The American Journal of Surgical Pathology*, *39*(12), 1730–1741. https://doi.org/10.1097/PAS.0000000000000533

Bengoechea-Alonso, M. T., & Ericsson, J. (2010a). The ubiquitin ligase Fbxw7 controls adipocyte differentiation by targeting C/EBPα for degradation. *Proceedings of the National Academy of Sciences*, *107*(26), 11817–11822. https://doi.org/10.1073/pnas.0913367107

Bengoechea-Alonso, M. T., & Ericsson, J. (2010b). Tumor suppressor Fbxw7 regulates TGFβ signaling by targeting TGIF1 for degradation. *Oncogene*, *29*(38), 5322–5328. https://doi.org/10.1038/onc.2010.278

Bischoff, F. R., & Ponstingl, H. (1991). Catalysis of guanine nucleotide exchange on Ran by the mitotic regulator RCC1. *Nature*, *354*(6348), 80–82. https://doi.org/10.1038/354080a0

Biswas, M., Phan, D., Watanabe, M., & Chan, J. Y. (2011). The Fbw7 Tumor Suppressor Regulates Nuclear Factor E2-related Factor 1 Transcription Factor Turnover through Proteasome-mediated Proteolysis *. *Journal of Biological Chemistry*, *286*(45), 39282–39289. https://doi.org/10.1074/jbc.M111.253807

Boj, S. F., Hwang, C. I., Baker, L. A., Chio, I. I. C., Engle, D. D., Corbo, V., Jager, M., Ponz-Sarvise, M., Tiriac, H., Spector, M. S., Gracanin, A., Oni, T., Yu, K. H., Van Boxtel, R., Huch, M., Rivera, K. D., Wilson, J. P., Feigin, M. E., Öhlund, D., … Tuveson, D. A. (2015). Organoid models of human and mouse ductal pancreatic cancer. *Cell*, *160*(1–2), 324–338. https://doi.org/10.1016/J.CELL.2014.12.021

Bonetti, P., Davoli, T., Sironi, C., Amati, B., Pelicci, P. G., & Colombo, E. (2008). Nucleophosmin and its AML-associated mutant regulate c-Myc turnover through Fbw7 gamma. *The Journal of Cell Biology*, *182*(1), 19–26. https://doi.org/10.1083/jcb.200711040

Bramhall, S. R., Allum, W. H., Jones, A. G., Allwood, A., Cummins, C., & Neoptolemos, J. P. (1995). Treatment and survival in 13,560 patients with pancreatic cancer, and incidence of the disease, in the West Midlands: An epidemiological study. *The British Journal of Surgery*, *82*(1), 111–115. https://doi.org/10.1002/BJS.1800820137

Brito, D. A., & Rieder, C. L. (2006). Mitotic Checkpoint Slippage in Humans Occurs via Cyclin B Destruction in the Presence of an Active Checkpoint. *Current Biology : CB*, *16*(12), 1194–1200. https://doi.org/10.1016/j.cub.2006.04.043

Bryant, H. E., Schultz, N., Thomas, H. D., Parker, K. M., Flower, D., Lopez, E., Kyle, S., Meuth, M., Curtin, N. J., & Helleday, T. (2005). Specific killing of BRCA2-deficient tumours with inhibitors of poly(ADP-ribose) polymerase. *Nature*, *434*(7035), 913–917. https://doi.org/10.1038/nature03443

Busino, L., Donzelli, M., Chiesa, M., Guardavaccaro, D., Ganoth, D., Dorrello, N. V., Hershko, A., Pagano, M., & Draetta, G. F. (2003). Degradation of Cdc25A by beta-TrCP during S phase and in response to DNA damage. *Nature*, *426*(6962), 87–91. https://doi.org/10.1038/nature02082

Caldas, C., Hahn, S. A., Da-Costa, L. T., Redston, M. S., Schutte, M., Seymour, A. B., Weinstein, C. L., Hruban, R. H., Yeo, C. J., & Kern, S. E. (1994). Frequent somatic mutations and homozygous deletions of the p16 (MTS1) gene in pancreatic adenocarcinoma. *Nature Genetics 1994 8:1*, *8*(1), 27–32. https://doi.org/10.1038/NG0994-27

Calhoun, E. S., Jones, J. B., Ashfaq, R., Adsay, V., Baker, S. J., Valentine, V., Hempen, P. M., Hilgers, W., Yeo, C. J., Hruban, R. H., & Kern, S. E. (2003). BRAF and FBXW7 (CDC4, FBW7, AGO, SEL10) mutations in distinct subsets of pancreatic cancer: Potential therapeutic targets. *The American Journal of Pathology*, *163*(4), 1255–1260. https://doi.org/10.1016/S0002-9440(10)63485-2

Campbell, P. J., Yachida, S., Mudie, L. J., Stephens, P. J., Pleasance, E. D., Stebbings, L. A., Morsberger, L. A., Latimer, C., McLaren, S., Lin, M. L., McBride, D. J., Varela, I., Nik-Zainal, S. A., Leroy, C., Jia, M., Menzies, A., Butler, A. P., Teague, J. W., Griffin, C. A., … Futreal, P. A. (2010). The patterns and dynamics of genomic instability in metastatic pancreatic cancer. *Nature*, *467*(7319), 1109–1113. https://doi.org/10.1038/nature09460

Cassavaugh, J. M., Hale, S. A., Wellman, T. L., Howe, A. K., Wong, C., & Lounsbury, K. M. (2011). Negative regulation of HIF-1α by an FBW7-mediated degradation pathway during hypoxia. *Journal of Cellular Biochemistry*, *112*(12), 3882–3890. https://doi.org/10.1002/jcb.23321

Cenciarelli, C., Chiaur, D. S., Guardavaccaro, D., Parks, W., Vidal, M., & Pagano, M. (1999). Identification of a family of human F-box proteins. *Current Biology*, *9*(20), 1177–1179. https://doi.org/10.1016/S0960-9822(00)80020-2

Chan, C.-H., Morrow, J. K., Li, C.-F., Gao, Y., Jin, G., Moten, A., Stagg, L. J., Ladbury, J. E., Cai, Z., Xu, D., Logothetis, C. J., Hung, M.-C., Zhang, S., & Lin, H.-K. (2013). Pharmacological inactivation of Skp2 SCF ubiquitin ligase restricts cancer stem cell traits and cancer progression. *Cell*, *154*(3), 556–568. https://doi.org/10.1016/j.cell.2013.06.048

Chari, S. T., Leibson, C. L., Rabe, K. G., Ransom, J., de Andrade, M., & Petersen, G. M. (2005). Probability of Pancreatic Cancer Following Diabetes: A Population-Based Study. *Gastroenterology*, *129*(2), 504–511. https://doi.org/10.1053/j.gastro.2005.05.007

Chatr-Aryamontri, A., Oughtred, R., Boucher, L., Rust, J., Chang, C., Kolas, N. K., O'Donnell, L., Oster, S., Theesfeld, C., Sellam, A., Stark, C., Breitkreutz, B.-J., Dolinski, K., & Tyers, M. (2017). The BioGRID interaction database: 2017 update. *Nucleic Acids Research*, *45*(D1), D369–D379. https://doi.org/10.1093/nar/gkw1102

Chen, J., Shin, J.-H., Zhao, R., Phan, L., Wang, H., Xue, Y., Post, S. M., Ho Choi, H., Chen, J.-S., Wang, E., Zhou, Z., Tseng, C., Gully, C., Velazquez-Torres, G., Fuentes-Mattei, E., Yeung, G., Qiao, Y., Chou, P.-C., Su, C.-H., … Lee, M.-H. (2014). CSN6 drives carcinogenesis by positively regulating Myc stability. *Nature Communications*, *5*, 5384. https://doi.org/10.1038/ncomms6384

Chen, M.-C., Chen, C.-H., Chuang, H.-C., Kulp, S. K., Teng, C.-M., & Chen, C.-S. (2011). Novel mechanism by which histone deacetylase inhibitors facilitate topoisomerase IIα degradation in hepatocellular carcinoma cells. *Hepatology*, *53*(1), 148–159. https://doi.org/10.1002/hep.23964

Chen, X., Li, X. Y., Long, M., Wang, X., Gao, Z. W., Cui, Y., Ren, J., Zhang, Z., Liu, C., Dong, K., & Zhang, H. (2018). The FBXW7 tumor suppressor inhibits breast cancer proliferation and promotes apoptosis by targeting MTDH for degradation. *Neoplasma*, *65*(2), 201–209. https://doi.org/10.4149/neo_2018_170228N149

Chen, Y., Li, Y., Xue, J., Gong, A., Yu, G., Zhou, A., Lin, K., Zhang, S., Zhang, N., Gottardi, C. J., & Huang, S. (2016). Wnt-induced deubiquitination FoxM1 ensures nucleus β-catenin transactivation. *The EMBO Journal*, *35*(6), 668–684. https://doi.org/10.15252/embj.201592810

Cheng, X., Hao, Y., Shu, W., Zhao, M., Zhao, C., Wu, Y., Peng, X., Yao, P., Xiao, D., Qing, G., Pan, Z., Yin, L., Hu, D., & Du, H.-N. (2017). Cell cycle-dependent degradation of the methyltransferase SETD3 attenuates cell proliferation and liver tumorigenesis. *The Journal of Biological Chemistry*, *292*(22), 9022–9033. https://doi.org/10.1074/jbc.M117.778001

Chiou, S. H., Risca, V. I., Wang, G. X., Yang, D., Gruner, B. M., Kathiria, A. S., Ma, R. K., Vaka, D., Chu, P., Kozak, M., Castellini, L., Graves, E. E., Kim, G. E., Mourrain, P., Koong, A. C., Giaccia, A. J., & Winslow, M. M. (2017). BLIMP1 induces transient metastatic heterogeneity

in pancreatic cancer. *Cancer Discovery*, *7*(10), 1184–1199. https://doi.org/10.1158/2159-8290.CD-17-0250

Chiou, S. H., Winters, I. P., Wang, J., Naranjo, S., Dudgeon, C., Tamburini, F. B., Brady, J. J., Yang, D., Grüner, B. M., Chuang, C. H., Caswell, D. R., Zeng, H., Chu, P., Kim, G. E., Carpizo, D. R., Kim, S. K., & Winslow, M. M. (2015). Pancreatic cancer modeling using retrograde viral vector delivery and in vivo CRISPR/Cas9-mediated somatic genome editing. *Genes & Development*, *29*(14), 1576–1585. https://doi.org/10.1101/GAD.264861.115

Ciechanover, A. (2005). Proteolysis: From the lysosome to ubiquitin and the proteasome. *Nature Reviews. Molecular Cell Biology*, *6*(1), 79–87. https://doi.org/10.1038/nrm1552

Ciechanover, A. (2015). The unravelling of the ubiquitin system. *Nature Reviews Molecular Cell Biology*, *16*(5), 322–324. https://doi.org/10.1038/nrm3982

Ciechanover, A., Elias, S., Heller, H., & Hershko, A. (1982). "Covalent affinity" purification of ubiquitin-activating enzyme. *The Journal of Biological Chemistry*, *257*(5), 2537–2542.

Ciechanover, A., Heller, H., Elias, S., Haas, A. L., & Hershko, A. (1980). ATP-dependent conjugation of reticulocyte proteins with the polypeptide required for protein degradation. *Proceedings of the National Academy of Sciences of the United States of America*, *77*(3), 1365–1368. https://doi.org/10.1073/pnas.77.3.1365

Ciechanover, A., Heller, H., Katz-Etzion, R., & Hershko, A. (1981). Activation of the heat-stable polypeptide of the ATP-dependent proteolytic system. *Proceedings of the National Academy of Sciences of the United States of America*, *78*(2), 761–765.

Ciehanover, A., Hod, Y., & Hershko, A. (1978). A heat-stable polypeptide component of an ATP-dependent proteolytic system from reticulocytes. *Biochemical and Biophysical Research Communications*, *81*(4), 1100–1105. https://doi.org/10.1016/0006-291x(78)91249-4

Classification of PanIN Lesions in the Pancreas—Pancreatic Cancer | Johns Hopkins Pathology. (n.d.). Retrieved June 23, 2022, from https://pathology.jhu.edu/pancreas/medical-professionals/duct-lesions

Collet, L., Ghurburrun, E., Meyers, N., Assi, M., Pirlot, B., Leclercq, I. A., Couvelard, A., Komuta, M., Cros, J., Demetter, P., Lemaigre, F. P., Borbath, I., & Jacquemin, P. (2020). Kras and Lkb1 mutations synergistically induce intraductal papillary mucinous neoplasm derived from pancreatic duct cells. *Gut*, *69*(4), 704–714. https://doi.org/10.1136/GUTJNL-2018-318059

Collisson, E. A., Trejo, C. L., Silva, J. M., Gu, S., Korkola, J. E., Heiser, L. M., Charles, R. P., Rabinovich, B. A., Hann, B., Dankort, D., Spellman, P. T., Phillips, W. A., Gray, J. W., & McMahon, M. (2012). A Central role for RAF→MEK→ERK signaling in the genesis of pancreatic ductal adenocarcinoma. *Cancer Discovery*, *2*(8), 685–693. https://doi.org/10.1158/2159-8290.CD-11-0347/42120/AM/A-CENTRAL-ROLE-FOR-RAF-MEK-ERK-SIGNALING-IN-THE

Cote, G. A., Gore, A. J., McElyea, S. D., Heathers, L. E., Xu, H., Sherman, S., & Korc, M. (2014). A pilot study to develop a diagnostic test for pancreatic ductal adenocarcinoma based on differential expression of select miRNA in plasma and bile. *The American Journal of Gastroenterology*, *109*(12), 1942–1952. https://doi.org/10.1038/AJG.2014.331

Cova, C. de la, & Greenwald, I. (2012). SEL-10/Fbw7-dependent negative feedback regulation of LIN-45/Braf signaling in C. elegans via a conserved phosphodegron. *Genes & Development*, *26*(22), 2524–2535. https://doi.org/10.1101/gad.203703.112

Cui, D., Xiong, X., Shu, J., Dai, X., Sun, Y., & Zhao, Y. (2020). FBXW7 Confers Radiation Survival by Targeting p53 for Degradation. *Cell Reports*, *30*(2), 497-509.e4. https://doi.org/10.1016/j.celrep.2019.12.032

Dai, X., North, B. J., & Inuzuka, H. (2014). Negative regulation of DAB2IP by Akt and SCF Fbw7 pathways. *Oncotarget*, *5*(10), 3307–3315. https://doi.org/10.18632/oncotarget.1939

Davis, M. A., Larimore, E. A., Fissel, B. M., Swanger, J., Taatjes, D. J., & Clurman, B. E. (2013). The SCF–Fbw7 ubiquitin ligase degrades MED13 and MED13L and regulates CDK8 module association with Mediator. *Genes & Development*, *27*(2), 151–156. https://doi.org/10.1101/gad.207720.112

Davis, R. J., Swanger, J., Hughes, B. T., & Clurman, B. E. (2017). The PP2A-B56 Phosphatase Opposes Cyclin E Autocatalytic Degradation via Site-Specific Dephosphorylation. *Molecular and Cellular Biology*, *37*(8), e00657-16. https://doi.org/10.1128/MCB.00657-16

Davis, R. J., Welcker, M., & Clurman, B. E. (2014). Tumor suppression by the Fbw7 ubiquitin ligase: Mechanisms and opportunities. *Cancer Cell*, *26*(4), 455–464. https://doi.org/10.1016/j.ccell.2014.09.013

Deng, L., Meng, T., Chen, L., Wei, W., & Wang, P. (2020). The role of ubiquitination in tumorigenesis and targeted drug discovery. *Signal Transduction and Targeted Therapy*, *5*(1), 1–28. https://doi.org/10.1038/s41392-020-0107-0

Dice, J. F., Chiang, H. L., Spencer, E. P., & Backer, J. M. (1986). Regulation of catabolism of microinjected ribonuclease A. Identification of residues 7-11 as the essential pentapeptide. *The Journal of Biological Chemistry*, *261*(15), 6853–6859.

Driscoll, J., & Goldberg, A. L. (1990). The proteasome (multicatalytic protease) is a component of the 1500-kDa proteolytic complex which degrades ubiquitin-conjugated proteins. *The Journal of Biological Chemistry*, *265*(9), 4789–4792.

Drosos, Y., Escobar, D., Chiang, M. Y., Roys, K., Valentine, V., Valentine, M. B., Rehg, J. E., Sahai, V., Begley, L. A., Ye, J., Paul, L., McKinnon, P. J., & Sosa-Pineda, B. (2017). ATM-deficiency increases genomic instability and metastatic potential in a mouse model of pancreatic cancer. *Scientific Reports 2017 7:1*, *7*(1), 1–14. https://doi.org/10.1038/s41598-017-11661-8

Durgan, J., & Parker, P. J. (2010). Regulation of the tumour suppressor Fbw7α by PKC-dependent phosphorylation and cancer-associated mutations. *The Biochemical Journal*, *432*(1), 77–87. https://doi.org/10.1042/BJ20100799

Duve, C. D., Gianetto, R., Appelmans, F., & Wattiaux, R. (1953). Enzymic Content of the Mitochondria Fraction. *Nature*, *172*(4390), 1143–1144. https://doi.org/10.1038/1721143a0

Ellis, C., Ramzy, A., & Kieffer, T. J. (2017). Regenerative medicine and cell-based approaches to restore pancreatic function. *Nature Reviews Gastroenterology & Hepatology*, *14*(10), 612–628. https://doi.org/10.1038/nrgastro.2017.93

Erhardt, J. A., Hynicka, W., DiBenedetto, A., Shen, N., Stone, N., Paulson, H., & Pittman, R. N. (1998). A novel F box protein, NFB42, is highly enriched in neurons and induces growth arrest. *The Journal of Biological Chemistry*, *273*(52), 35222–35227. https://doi.org/10.1074/jbc.273.52.35222

Etlinger, J. D., & Goldberg, A. L. (1977). A Soluble ATP-Dependent Proteolytic System Responsible for the Degradation of Abnormal Proteins in Reticulocytes. *Proceedings of the National Academy of Sciences of the United States of America*, *74*(1), 54–58.

Eytan, E., Ganoth, D., Armon, T., & Hershko, A. (1989). ATP-dependent incorporation of 20S protease into the 26S complex that degrades proteins conjugated to ubiquitin. *Proceedings of the National Academy of Sciences of the United States of America*, *86*(20), 7751–7755.

Fang, L., Yang, Z., Zhou, J., Tung, J.-Y., Hsiao, C.-D., Wang, L., Deng, Y., Wang, P., Wang, J., & Lee, M.-H. (2015). Circadian Clock Gene CRY2 Degradation Is Involved in Chemoresistance of Colorectal Cancer. *Molecular Cancer Therapeutics*, *14*(6), 1476–1487. https://doi.org/10.1158/1535-7163.MCT-15-0030

Fernandez, P. C., Frank, S. R., Wang, L., Schroeder, M., Liu, S., Greene, J., Cocito, A., & Amati, B. (2003). Genomic targets of the human c-Myc protein. *Genes & Development*, *17*(9), 1115–1129. https://doi.org/10.1101/gad.1067003

Foltz, D. R., Santiago, M. C., Berechid, B. E., & Nye, J. S. (2002). Glycogen synthase kinase-3beta modulates notch signaling and stability. *Current Biology: CB*, *12*(12), 1006–1011. https://doi.org/10.1016/s0960-9822(02)00888-6

Forbes, S. A., Beare, D., Boutselakis, H., Bamford, S., Bindal, N., Tate, J., Cole, C. G., Ward, S., Dawson, E., Ponting, L., Stefancsik, R., Harsha, B., Kok, C. Y., Jia, M., Jubb, H., Sondka, Z., Thompson, S., De, T., & Campbell, P. J. (2017). COSMIC: Somatic cancer genetics at high-resolution. *Nucleic Acids Research*, *45*(D1), D777–D783. https://doi.org/10.1093/nar/gkw1121

Frappart, P.-O., & Hofmann, T. G. (2020). Pancreatic Ductal Adenocarcinoma (PDAC) Organoids: The Shining Light at the End of the Tunnel for Drug Response Prediction and Personalized Medicine. *Cancers*, *12*(10). https://doi.org/10.3390/cancers12102750

Galli, F., Rossi, M., D'Alessandra, Y., De Simone, M., Lopardo, T., Haupt, Y., Alsheich-Bartok, O., Anzi, S., Shaulian, E., Calabrò, V., La Mantia, G., & Guerrini, L. (2010). MDM2 and Fbw7 cooperate to induce p63 protein degradation following DNA damage and cell differentiation. *Journal of Cell Science*, *123*(14), 2423–2433. https://doi.org/10.1242/jcs.061010

Gao, J., Azmi, A. S., Aboukameel, A., Kauffman, M., Shacham, S., Abou-Samra, A.-B., & Mohammad, R. M. (2014). Nuclear retention of Fbw7 by specific inhibitors of nuclear export leads to Notch1 degradation in pancreatic cancer. *Oncotarget*, *5*(11), 3444–3454. https://doi.org/10.18632/oncotarget.1813

García-Higuera, I., Manchado, E., Dubus, P., Cañamero, M., Méndez, J., Moreno, S., & Malumbres, M. (2008). Genomic stability and tumour suppression by the APC/C cofactor Cdh1. *Nature Cell Biology*, *10*(7), 802–811. https://doi.org/10.1038/ncb1742

Garvin, A. J., Khalaf, A. H. A., Rettino, A., Xicluna, J., Butler, L., Morris, J. R., Heery, D. M., & Clarke, N. M. (2019). GSK3β-SCFFBXW7α mediated phosphorylation and ubiquitination of IRF1 are required for its transcription-dependent turnover. *Nucleic Acids Research*, *47*(9), 4476–4494. https://doi.org/10.1093/nar/gkz163

Gasser, J. A., Inuzuka, H., Lau, A. W., Wei, W., Beroukhim, R., & Toker, A. (2014). SGK3 Mediates INPP4B-Dependent PI3K Signaling in Breast Cancer. *Molecular Cell*, *56*(4), 595–607. https://doi.org/10.1016/j.molcel.2014.09.023

Geng, F., Wenzel, S., & Tansey, W. P. (2012). Ubiquitin and proteasomes in transcription. *Annual Review of Biochemistry*, *81*, 177–201. https://doi.org/10.1146/annurev-biochem-052110-120012

Geng, L., Huntoon, C. J., & Karnitz, L. M. (2010). RAD18-mediated ubiquitination of PCNA activates the Fanconi anemia DNA repair network. *The Journal of Cell Biology*, *191*(2), 249–257. https://doi.org/10.1083/jcb.201005101

Genkinger, J. M., Kitahara, C. M., Bernstein, L., Berrington de Gonzalez, A., Brotzman, M., Elena, J. W., Giles, G. G., Hartge, P., Singh, P. N., Stolzenberg-Solomon, R. Z., Weiderpass, E., Adami, H.-O., Anderson, K. E., Beane-Freeman, L. E., Buring, J. E., Fraser, G. E., Fuchs, C. S., Gapstur, S. M., Gaziano, J. M., … Jacobs, E. J. (2015). Central adiposity, obesity during early adulthood, and pancreatic cancer mortality in a pooled analysis of cohort studies. *Annals of Oncology : Official Journal of the European Society for Medical Oncology*, *26*(11), 2257–2266. https://doi.org/10.1093/annonc/mdv355

Gianetto, R., & De Duve, C. (1955). Tissue fractionation studies. 4. Comparative study of the binding of acid phosphatase, β-glucuronidase and cathepsin by rat-liver particles. *Biochemical Journal*, *59*(3), 433–438.

Giráldez, S., Herrero-Ruiz, J., Mora-Santos, M., Japón, M. Á., Tortolero, M., & Romero, F. (2014). SCF FBXW7α modulates the intra-S-phase DNA-damage checkpoint by regulating Polo like kinase-1 stability. *Oncotarget*, *5*(12), 4370–4383. https://doi.org/10.18632/oncotarget.2021

Goldberg, A. L. (2013). *Protein Degradation*. https://doi.org/10.1016/B978-0-12-378630-2.00063-3

Goldberg, A. L., & Dice, J. F. (1974). Intracellular protein degradation in mammalian and bacterial cells. *Annual Review of Biochemistry*, *43*(0), 835–869. https://doi.org/10.1146/annurev.bi.43.070174.004155

Goldberg, A. L., & St John, A. C. (1976). Intracellular protein degradation in mammalian and bacterial cells: Part 2. *Annual Review of Biochemistry*, *45*, 747–803. https://doi.org/10.1146/annurev.bi.45.070176.003531

Gossage, L., Eisen, T., & Maher, E. R. (2015). VHL, the story of a tumour suppressor gene. *Nature Reviews. Cancer*, *15*(1), 55–64. https://doi.org/10.1038/nrc3844

Greil, C., Krohs, J., Schnerch, D., Follo, M., Felthaus, J., Engelhardt, M., & Wäsch, R. (2016). The role of APC/C(Cdh1) in replication stress and origin of genomic instability. *Oncogene*, *35*(23), 3062–3070. https://doi.org/10.1038/onc.2015.367

Grim, J. E., Gustafson, M. P., Hirata, R. K., Hagar, A. C., Swanger, J., Welcker, M., Hwang, H. C., Ericsson, J., Russell, D. W., & Clurman, B. E. (2008). Isoform- and cell cycle-dependent substrate degradation by the Fbw7 ubiquitin ligase. *The Journal of Cell Biology*, *181*(6), 913–920. https://doi.org/10.1083/jcb.200802076

Guerra, C., Schuhmacher, A. J., Cañamero, M., Grippo, P. J., Verdaguer, L., Pérez-Gallego, L., Dubus, P., Sandgren, E. P., & Barbacid, M. (2007). Chronic Pancreatitis Is Essential for Induction of Pancreatic Ductal Adenocarcinoma by K-Ras Oncogenes in Adult Mice. *Cancer Cell*, *11*(3), 291–302. https://doi.org/10.1016/J.CCR.2007.01.012/ATTACHMENT/F5A92F9A-CA03-454C-B2E1-787B9B8050B9/MMC1.PDF

Haas, A. L., & Rose, I. A. (1982). The mechanism of ubiquitin activating enzyme. A kinetic and equilibrium analysis. *The Journal of Biological Chemistry*, *257*(17), 10329–10337.

Haeberle, L., & Esposito, I. (2019). Pathology of pancreatic cancer. *Translational Gastroenterology and Hepatology*, *4*(June). https://doi.org/10.21037/TGH.2019.06.02

Hao, B., Oehlmann, S., Sowa, M. E., Harper, J. W., & Pavletich, N. P. (2007). Structure of a Fbw7-Skp1-cyclin E complex: Multisite-phosphorylated substrate recognition by SCF ubiquitin ligases. *Molecular Cell*, *26*(1), 131–143. https://doi.org/10.1016/j.molcel.2007.02.022

He, L., Torres-Lockhart, K., Forster, N., Ramakrishnan, S., Greninger, P., Garnett, M. J., McDermott, U., Rothenberg, S. M., Benes, C. H., & Ellisen, L. W. (2013). Mcl-1 and FBW7

control a dominant survival pathway underlying HDAC and Bcl-2 inhibitor synergy in squamous cell carcinoma. *Cancer Discovery*, *3*(3), 324–337. https://doi.org/10.1158/2159-8290.CD-12-0417

Hershko, A., Ciechanover, A., Heller, H., Haas, A. L., & Rose, I. A. (1980). Proposed role of ATP in protein breakdown: Conjugation of protein with multiple chains of the polypeptide of ATP-dependent proteolysis. *Proceedings of the National Academy of Sciences of the United States of America*, *77*(4), 1783–1786. https://doi.org/10.1073/pnas.77.4.1783

Hershko, A., Ciechanover, A., & Rose, I. A. (1979). Resolution of the ATP-dependent proteolytic system from reticulocytes: A component that interacts with ATP. *Proceedings of the National Academy of Sciences of the United States of America*, *76*(7), 3107–3110.

Hershko, A., Ciechanover, A., & Rose, I. A. (1981). Identification of the active amino acid residue of the polypeptide of ATP-dependent protein breakdown. *The Journal of Biological Chemistry*, *256*(4), 1525–1528.

Hershko, A., Leshinsky, E., Ganoth, D., & Heller, H. (1984). ATP-dependent degradation of ubiquitin-protein conjugates. *Proceedings of the National Academy of Sciences of the United States of America*, *81*(6), 1619–1623.

Hershko, A., & Tomkins, G. M. (1971). Studies on the degradation of tyrosine aminotransferase in hepatoma cells in culture. Influence of the composition of the medium and adenosine triphosphate dependence. *The Journal of Biological Chemistry*, *246*(3), 710–714.

Hidalgo, M. (2010). Pancreatic Cancer. *New England Journal of Medicine*, *362*(17), 1605–1617. https://doi.org/10.1056/NEJMra0901557

Hindley, C. J., Cordero-Espinoza, L., & Huch, M. (2016). Organoids from adult liver and pancreas: Stem cell biology and biomedical utility. *Developmental Biology*, *420*(2), 251–261. https://doi.org/10.1016/J.YDBIO.2016.06.039

Hingorani, S. R., Petricoin, E. F., Maitra, A., Rajapakse, V., King, C., Jacobetz, M. A., Ross, S., Conrads, T. P., Veenstra, T. D., Hitt, B. A., Kawaguchi, Y., Johann, D., Liotta, L. A., Crawford, H. C., Putt, M. E., Jacks, T., Wright, C. V. E., Hruban, R. H., Lowy, A. M., & Tuveson, D. A. (2003a). Preinvasive and invasive ductal pancreatic cancer and its early detection in the mouse. *Cancer Cell*, *4*(6), 437–450. https://doi.org/10.1016/S1535-6108(03)00309-X

Hingorani, S. R., Petricoin, E. F., Maitra, A., Rajapakse, V., King, C., Jacobetz, M. A., Ross, S., Conrads, T. P., Veenstra, T. D., Hitt, B. A., Kawaguchi, Y., Johann, D., Liotta, L. A., Crawford, H. C., Putt, M. E., Jacks, T., Wright, C. V. E., Hruban, R. H., Lowy, A. M., & Tuveson, D. A. (2003b). Preinvasive and invasive ductal pancreatic cancer and its early detection in the mouse. *Cancer Cell*, *4*(6), 437–450. https://doi.org/10.1016/S1535-6108(03)00309-X/ATTACHMENT/E4365F87-FD7D-4C83-B8BD-E8C99A4B7F21/MMC1.PDF

Hingorani, S. R., Wang, L., Multani, A. S., Combs, C., Deramaudt, T. B., Hruban, R. H., Rustgi, A. K., Chang, S., & Tuveson, D. A. (2005). Trp53R172H and KrasG12D cooperate to promote chromosomal instability and widely metastatic pancreatic ductal adenocarcinoma in mice. *Cancer Cell*, *7*(5), 469–483. https://doi.org/10.1016/J.CCR.2005.04.023

Hoffman, L., Pratt, G., & Rechsteiner, M. (1992). Multiple forms of the 20 S multicatalytic and the 26 S ubiquitin/ATP-dependent proteases from rabbit reticulocyte lysate. *Journal of Biological Chemistry*, *267*(31), 22362–22368. https://doi.org/10.1016/S0021-9258(18)41680-8

Hogness, D. S., Cohn, M., & Monod, J. (1955). Studies on the induced synthesis of beta-galactosidase in Escherichia coli: The kinetics and mechanism of sulfur incorporation.

Biochimica Et Biophysica Acta, *16*(1), 99–116. https://doi.org/10.1016/0006-3002(55)90188-8

Hong, X., Liu, W., Song, R., Shah, J. J., Feng, X., Tsang, C. K., Morgan, K. M., Bunting, S. F., Inuzuka, H., Zheng, X. F. S., Shen, Z., Sabaawy, H. E., Liu, L., & Pine, S. R. (2016). SOX9 is targeted for proteasomal degradation by the E3 ligase FBW7 in response to DNA damage. *Nucleic Acids Research*, *44*(18), 8855–8869. https://doi.org/10.1093/nar/gkw748

Hoogstraat, M., de Pagter, M. S., Cirkel, G. A., van Roosmalen, M. J., Harkins, T. T., Duran, K., Kreeftmeijer, J., Renkens, I., Witteveen, P. O., Lee, C. C., Nijman, I. J., Guy, T., van 't Slot, R., Jonges, T. N., Lolkema, M. P., Koudijs, M. J., Zweemer, R. P., Voest, E. E., Cuppen, E., & Kloosterman, W. P. (2014). Genomic and transcriptomic plasticity in treatment-naive ovarian cancer. *Genome Research*, *24*(2), 200–211. https://doi.org/10.1101/gr.161026.113

Hough, R., Pratt, G., & Rechsteiner, M. (1986). Ubiquitin-lysozyme conjugates. Identification and characterization of an ATP-dependent protease from rabbit reticulocyte lysates. *Journal of Biological Chemistry*, *261*(5), 2400–2408. https://doi.org/10.1016/S0021-9258(17)35950-1

Hough, R., Pratt, G., & Rechsteiner, M. (1987). Purification of two high molecular weight proteases from rabbit reticulocyte lysate. *The Journal of Biological Chemistry*, *262*(17), 8303–8313.

Housley, M. P., Udeshi, N. D., Rodgers, J. T., Shabanowitz, J., Puigserver, P., Hunt, D. F., & Hart, G. W. (2009). A PGC-1α-O-GlcNAc Transferase Complex Regulates FoxO Transcription Factor Activity in Response to Glucose. *The Journal of Biological Chemistry*, *284*(8), 5148–5157. https://doi.org/10.1074/jbc.M808890200

Hruban, R. H., Adsay, N. V., Albores-Saavedra, J., Compton, C., Garrett, E. S., Goodman, S. N., Kern, S. E., Klimstra, D. S., Klöppel, G., Longnecker, D. S., Lüttges, J., & Offerhaus, G. J. A. (2001). Pancreatic intraepithelial neoplasia: A new nomenclature and classification system for pancreatic duct lesions. *American Journal of Surgical Pathology*, *25*(5), 579–586. https://doi.org/10.1097/00000478-200105000-00003

Hu, H. feng, Ye, Z., Qin, Y., Xu, X. wu, Yu, X. jun, Zhuo, Q. feng, & Ji, S. rong. (2021). Mutations in key driver genes of pancreatic cancer: Molecularly targeted therapies and other clinical implications. *Acta Pharmacologica Sinica 2021 42:11*, *42*(11), 1725–1741. https://doi.org/10.1038/S41401-020-00584-2

Huang, D. T., Hunt, H. W., Zhuang, M., Ohi, M. D., Holton, J. M., & Schulman, B. A. (2007). Basis for a ubiquitin-like protein thioester switch toggling E1-E2 affinity. *Nature*, *445*(7126), 394–398. https://doi.org/10.1038/nature05490

Huang, D. T., Paydar, A., Zhuang, M., Waddell, M. B., Holton, J. M., & Schulman, B. A. (2005). Structural basis for recruitment of Ubc12 by an E2 binding domain in NEDD8's E1. *Molecular Cell*, *17*(3), 341–350. https://doi.org/10.1016/j.molcel.2004.12.020

Huang, H.-L., Weng, H.-Y., Wang, L.-Q., Yu, C.-H., Huang, Q.-J., Zhao, P.-P., Wen, J.-Z., Zhou, H., & Qu, L.-H. (2012). Triggering Fbw7-mediated proteasomal degradation of c-Myc by oridonin induces cell growth inhibition and apoptosis. *Molecular Cancer Therapeutics*, *11*(5), 1155–1165. https://doi.org/10.1158/1535-7163.MCT-12-0066

Huang, L., Kinnucan, E., Wang, G., Beaudenon, S., Howley, P. M., Huibregtse, J. M., & Pavletich, N. P. (1999). Structure of an E6AP-UbcH7 complex: Insights into ubiquitination by the E2-E3 enzyme cascade. *Science (New York, N.Y.)*, *286*(5443), 1321–1326. https://doi.org/10.1126/science.286.5443.1321

Huang, L.-Y., Zhao, J., Chen, H., Wan, L., Inuzuka, H., Guo, J., Fu, X., Zhai, Y., Lu, Z., Wang, X., Han, Z.-G., Sun, Y., & Wei, W. (2018). SCFFBW7-mediated degradation of Brg1 suppresses gastric cancer metastasis. *Nature Communications*, *9*(1), 3569. https://doi.org/10.1038/s41467-018-06038-y

Huch, M., Bonfanti, P., Boj, S. F., Sato, T., Loomans, C. J. M., Van De Wetering, M., Sojoodi, M., Li, V. S. W., Schuijers, J., Gracanin, A., Ringnalda, F., Begthel, H., Hamer, K., Mulder, J., Van Es, J. H., De Koning, E., Vries, R. G. J., Heimberg, H., & Clevers, H. (2013). Unlimited in vitro expansion of adult bi-potent pancreas progenitors through the Lgr5/R-spondin axis. *The EMBO Journal*, *32*(20), 2708–2721. https://doi.org/10.1038/EMBOJ.2013.204

Inuzuka, H., Shaik, S., Onoyama, I., Gao, D., Tseng, A., Maser, R. S., Zhai, B., Wan, L., Gutierrez, A., Lau, A. W., Xiao, Y., Christie, A. L., Aster, J., Settleman, J., Gygi, S. P., Kung, A. L., Look, T., Nakayama, K. I., DePinho, R. A., & Wei, W. (2011). SCF(FBW7) regulates cellular apoptosis by targeting MCL1 for ubiquitylation and destruction. *Nature*, *471*(7336), 104–109. https://doi.org/10.1038/nature09732

Inuzuka, H., Tseng, A., Gao, D., Zhai, B., Zhang, Q., Shaik, S., Wan, L., Ang, X. L., Mock, C., Yin, H., Stommel, J. M., Gygi, S., Lahav, G., Asara, J., Xiao, Z.-X. J., Kaelin, W. G., Harper, J. W., & Wei, W. (2010). Phosphorylation by casein kinase I promotes the turnover of the Mdm2 oncoprotein via the SCF(beta-TRCP) ubiquitin ligase. *Cancer Cell*, *18*(2), 147–159. https://doi.org/10.1016/j.ccr.2010.06.015

Ishii, N., Araki, K., Yokobori, T., Gantumur, D., Yamanaka, T., Altan, B., Tsukagoshi, M., Igarashi, T., Watanabe, A., Kubo, N., Hosouchi, Y., Kuwano, H., & Shirabe, K. (2017). Reduced FBXW7 expression in pancreatic cancer correlates with poor prognosis and chemotherapeutic resistance via accumulation of MCL1. *Oncotarget*, *8*(68), 112636–112646. https://doi.org/10.18632/oncotarget.22634

Izeradjene, K., Combs, C., Best, M., Gopinathan, A., Wagner, A., Grady, W. M., Deng, C. X., Hruban, R. H., Adsay, N. V., Tuveson, D. A., & Hingorani, S. R. (2007). Kras(G12D) and Smad4/Dpc4 haploinsufficiency cooperate to induce mucinous cystic neoplasms and invasive adenocarcinoma of the pancreas. *Cancer Cell*, *11*(3), 229–243. https://doi.org/10.1016/J.CCR.2007.01.017

Javeed, N., Sagar, G., Dutta, S. K., Smyrk, T. C., Lau, J. S., Bhattacharya, S., Truty, M., Petersen, G. M., Kaufman, R. J., Chari, S. T., & Mukhopadhyay, D. (2015). Pancreatic Cancer-Derived Exosomes Cause Paraneoplastic β-cell Dysfunction. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research*, *21*(7), 1722–1733. https://doi.org/10.1158/1078-0432.CCR-14-2022

Jesse Gore, A., Deitz, S. L., Palam, L. R., Craven, K. E., & Korc, M. (2014). Pancreatic cancer-associated retinoblastoma 1 dysfunction enables TGF-β to promote proliferation. *The Journal of Clinical Investigation*, *124*(1), 338–352. https://doi.org/10.1172/JCI71526

Jhon, P., & Donals, V. S. (1946). *Quantitative Clinical Chemistry: Interpretations.: Vol. I* (2nd ed.). Baltimore: Williams & Wilkind. https://www.science.org/doi/10.1126/science.106.2758.454.b

Ji, S., Qin, Y., Shi, S., Liu, X., Hu, H., Zhou, H., Gao, J., Zhang, B., Xu, W., Liu, J., Liang, D., Liu, L., Liu, C., Long, J., & Zhou, H. (2015). ERK kinase phosphorylates and destabilizes the tumor suppressor FBW7 in pancreatic cancer. *Nature Publishing Group*, 561–573. https://doi.org/10.1038/cr.2015.30

Jiang, J.-X., Sun, C.-Y., Tian, S., Yu, C., Chen, M.-Y., & Zhang, H. (2016). Tumor suppressor Fbxw7 antagonizes WNT signaling by targeting β-catenin for degradation in pancreatic

cancer. *Tumour Biology: The Journal of the International Society for Oncodevelopmental Biology and Medicine*, *37*(10), 13893–13902. https://doi.org/10.1007/s13277-016-5217-5

Jin, X., Yang, C., Fan, P., Xiao, J., Zhang, W., Zhan, S., Liu, T., Wang, D., & Wu, H. (2017). CDK5/FBW7-dependent ubiquitination and degradation of EZH2 inhibits pancreatic cancer cell migration and invasion. *Journal of Biological Chemistry*, *292*(15), 6269–6280. https://doi.org/10.1074/jbc.M116.764407

Johnston, F. M., Tan, M. C. B., Tan, B. R., Porembka, M. R., Brunt, E. M., Linehan, D. C., Simon, P. O., Plambeck-Suess, S., Eberlein, T. J., Hellstrom, K. E., Hellstrom, I., Hawkins, W. G., & Goedegebuure, P. (2009). Circulating mesothelin protein and cellular antimesothelin immunity in patients with pancreatic cancer. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research*, *15*(21), 6511–6518. https://doi.org/10.1158/1078-0432.CCR-09-0565

JunGang, Z., Jun, T., WanFu, M., & KaiMing, R. (2012). FBXW7-mediated degradation of CCDC6 is impaired by ATM during DNA damage response in lung cancer cells. *FEBS Letters*, *586*(24), 4257–4263. https://doi.org/10.1016/j.febslet.2012.10.029

Junttila, M. R., & Evan, G. I. (2009). p53—A Jack of all trades but master of none. *Nature Reviews. Cancer*, *9*(11), 821–829. https://doi.org/10.1038/NRC2728

Kamadurai, H. B., Qiu, Y., Deng, A., Harrison, J. S., MacDonald, C., Actis, M., Rodrigues, P., Miller, D. J., Souphron, J., Lewis, S. M., Kurinov, I., Fujii, N., Hammel, M., Piper, R., Kuhlman, B., & Schulman, B. A. (2013). Mechanism of ubiquitin ligation and lysine prioritization by a HECT E3. *ELife*, *2*, e00828. https://doi.org/10.7554/eLife.00828

Kannan, M. B., Dodard-Friedman, I., & Blank, V. (2015). Stringent Control of NFE2L3 (Nuclear Factor, Erythroid 2-Like 3; NRF3) Protein Degradation by FBW7 (F-box/WD Repeat-containing Protein 7) and Glycogen Synthase Kinase 3 (GSK3). *The Journal of Biological Chemistry*, *290*(43), 26292–26302. https://doi.org/10.1074/jbc.M115.666446

Kawaguchi, Y., Cooper, B., Gannon, M., Ray, M., MacDonald, R. J., & Wright, C. V. E. (2002). The role of the transcriptional regulator Ptf1a in converting intestinal to pancreatic progenitors. *Nature Genetics*, *32*(1), 128–134. https://doi.org/10.1038/ng959

Kee, Y., Kim, J. M., D'Andrea, A. D., & D'Andrea, A. (2009). Regulated degradation of FANCM in the Fanconi anemia pathway during mitosis. *Genes & Development*, *23*(5), 555–560. https://doi.org/10.1101/gad.1761309

Kharat, S. S., Tripathi, V., Damodaran, A. P., Priyadarshini, R., Chandra, S., Tikoo, S., Nandhakumar, R., Srivastava, V., Priya, S., Hussain, M., Kaur, S., Fishman, J. B., & Sengupta, S. (2016). Mitotic phosphorylation of Bloom helicase at Thr182 is required for its proteasomal degradation and maintenance of chromosomal stability. *Oncogene*, *35*(8), 1025–1038. https://doi.org/10.1038/onc.2015.157

Kim, D. S., Zhang, W., Millman, S. E., Hwang, B. J., Kwon, S. J., Clayberger, C., Pagano, M., & Krensky, A. M. (2012). Fbw7γ-mediated degradation of KLF13 prevents RANTES expression in resting human but not murine T lymphocytes. *Blood*, *120*(8), 1658–1667. https://doi.org/10.1182/blood-2012-03-415968

Kim, W. Y., & Sharpless, N. E. (2006). The regulation of INK4/ARF in cancer and aging. *Cell*, *127*(2), 265–275. https://doi.org/10.1016/J.CELL.2006.10.003

Kimura, T., Gotoh, M., Nakamura, Y., & Arakawa, H. (2003). HCDC4b, a regulator of cyclin E, as a direct transcriptional target of p53. *Cancer Science*, *94*(5), 431–436. https://doi.org/10.1111/j.1349-7006.2003.tb01460.x

Kimura, Y., Fukuda, A., Ogawa, S., Maruno, T., Takada, Y., Tsuda, M., Hiramatsu, Y., Araki, O., Nagao, M., Yoshikawa, T., Ikuta, K., Yoshioka, T., Wang, Z., Akiyama, H., Wright, C. V., Takaori, K., Uemoto, S., Chiba, T., & Seno, H. (2018). ARID1A Maintains Differentiation of Pancreatic Ductal Cells and Inhibits Development of Pancreatic Ductal Adenocarcinoma in Mice. *Gastroenterology*, *155*(1), 194-209.e2. https://doi.org/10.1053/J.GASTRO.2018.03.039

Kitade, S., Onoyama, I., Kobayashi, H., Yagi, H., Yoshida, S., Kato, M., Tsunematsu, R., Asanoma, K., Sonoda, K., Wake, N., Hata, K., Nakayama, K. I., & Kato, K. (2016). FBXW7 is involved in the acquisition of the malignant phenotype in epithelial ovarian tumors. *Cancer Science*, *107*(10), 1399–1405. https://doi.org/10.1111/cas.13026

Kitagawa, K., Hiramatsu, Y., Uchida, C., Isobe, T., Hattori, T., Oda, T., Shibata, K., Nakamura, S., Kikuchi, A., & Kitagawa, M. (2009). Fbw7 promotes ubiquitin-dependent degradation of c-Myb: Involvement of GSK3-mediated phosphorylation of Thr-572 in mouse c-Myb. *Oncogene*, *28*(25), 2393–2405. https://doi.org/10.1038/onc.2009.111

Kitagawa, K., Shibata, K., Matsumoto, A., Matsumoto, M., Ohhata, T., Nakayama, K. I., Niida, H., & Kitagawa, M. (2014a). Fbw7 Targets GATA3 through Cyclin-Dependent Kinase 2-Dependent Proteolysis and Contributes to Regulation of T-Cell Development. *Molecular and Cellular Biology*, *34*(14), 2732–2744. https://doi.org/10.1128/MCB.01549-13

Kitagawa, K., Shibata, K., Matsumoto, A., Matsumoto, M., Ohhata, T., Nakayama, K. I., Niida, H., & Kitagawa, M. (2014b). Fbw7 Targets GATA3 through Cyclin-Dependent Kinase 2-Dependent Proteolysis and Contributes to Regulation of T-Cell Development. *Molecular and Cellular Biology*, *34*(14), 2732–2744. https://doi.org/10.1128/MCB.01549-13

Kleeff, J., Korc, M., Apte, M., La Vecchia, C., Johnson, C. D., Biankin, A. V., Neale, R. E., Tempero, M., Tuveson, D. A., Hruban, R. H., & Neoptolemos, J. P. (2016). Pancreatic cancer. *Nature Reviews Disease Primers*, *2*(1), 16022. https://doi.org/10.1038/nrdp.2016.22

Klein, A. P. (2021). Pancreatic cancer epidemiology: Understanding the role of lifestyle and inherited risk factors. *Nature Reviews Gastroenterology & Hepatology*, *18*(7), 493–502. https://doi.org/10.1038/s41575-021-00457-x

Klein, A. P., Brune, K. A., Petersen, G. M., Goggins, M., Tersmette, A. C., Offerhaus, G. J. A., Griffin, C., Cameron, J. L., Yeo, C. J., Kern, S., & Hruban, R. H. (2004). Prospective Risk of Pancreatic Cancer in Familial Pancreatic Cancer Kindreds. *Cancer Research*, *64*(7), 2634–2638. https://doi.org/10.1158/0008-5472.CAN-03-3823

Klotz, K., Cepeda, D., Tan, Y., Sun, D., Sangfelt, O., & Spruck, C. (2009). SCFFbxw7/hCdc4 targets cyclin E2 for ubiquitin-dependent proteolysis. *Experimental Cell Research*, *315*(11), 1832–1839. https://doi.org/10.1016/j.yexcr.2008.11.017

Koepp, D. M., Schaefer, L. K., Ye, X., Keyomarsi, K., Chu, C., Harper, J. W., & Elledge, S. J. (2001). Phosphorylation-dependent ubiquitination of cyclin E by the SCFFbw7 ubiquitin ligase. *Science (New York, N.Y.)*, *294*(5540), 173–177. https://doi.org/10.1126/science.1065203

Kong, Y., Sharma, R. B., Nwosu, B. U., & Alonso, L. C. (2016). Islet biology, the CDKN2A/B locus and type 2 diabetes risk. *Diabetologia*, *59*(8), 1579–1593. https://doi.org/10.1007/S00125-016-3967-7

Koo, J., Wu, X., Mao, Z., Khuri, F. R., & Sun, S.-Y. (2015). Rictor Undergoes Glycogen Synthase Kinase 3 (GSK3)-dependent, FBXW7-mediated Ubiquitination and Proteasomal Degradation. *The Journal of Biological Chemistry*, *290*(22), 14120–14129. https://doi.org/10.1074/jbc.M114.633057

Kopp, J. L., Dubois, C. L., Schaeffer, D. F., Samani, A., Taghizadeh, F., Cowan, R. W., Rhim, A. D., Stiles, B. L., Valasek, M., & Sander, M. (2018). Loss of Pten and Activation of Kras Synergistically Induce Formation of Intraductal Papillary Mucinous Neoplasia From Pancreatic Ductal Cells in Mice. *Gastroenterology*, *154*(5), 1509-1523.e5. https://doi.org/10.1053/J.GASTRO.2017.12.007

Kourtis, N., Moubarak, R. S., Aranda-Orgilles, B., Lui, K., Aydin, I. T., Trimarchi, T., Darvishian, F., Salvaggio, C., Zhong, J., Bhatt, K., Chen, E. I., Celebi, J. T., Lazaris, C., Tsirigos, A., Osman, I., Hernando, E., & Aifantis, I. (2015). FBXW7 modulates cellular stress response and metastatic potential through HSF1 post-translational modification. *Nature Cell Biology*, *17*(3), 322–332. https://doi.org/10.1038/ncb3121

Kraft, E., Stone, S. L., Ma, L., Su, N., Gao, Y., Lau, O.-S., Deng, X.-W., & Callis, J. (2005). Genome Analysis and Functional Characterization of the E2 and RING-Type E3 Ligase Ubiquitination Enzymes of Arabidopsis. *Plant Physiology*, *139*(4), 1597–1611. https://doi.org/10.1104/pp.105.067983

Kruiswijk, F., Yuniati, L., Magliozzi, R., Low, T. Y., Lim, R., Bolder, R., Mohammed, S., Proud, C. G., Heck, A. J. R., Pagano, M., & Guardavaccaro, D. (2012). Coupled activation and degradation of eEF2K regulates protein synthesis in response to genotoxic stress. *Science Signaling*, *5*(227), ra40. https://doi.org/10.1126/scisignal.2002718

Kuai, X., Li, L., Chen, R., Wang, K., Chen, M., Cui, B., Zhang, Y., Li, J., Zhu, H., Zhou, H., Huang, J., Qin, J., Wang, Z., Wei, W., & Gao, D. (2019). SCFFBXW7/GSK3β-Mediated GFI1 Degradation Suppresses Proliferation of Gastric Cancer Cells. *Cancer Research*, *79*(17), 4387–4398. https://doi.org/10.1158/0008-5472.CAN-18-4032

Kumar, Y., Shukla, N., Thacker, G., Kapoor, I., Lochab, S., Bhatt, M. L. B., Chattopadhyay, N., Sanyal, S., & Trivedi, A. K. (2016). Ubiquitin Ligase, Fbw7, Targets CDX2 for Degradation via Two Phosphodegron Motifs in a GSK3β-Dependent Manner. *Molecular Cancer Research: MCR*, *14*(11), 1097–1109. https://doi.org/10.1158/1541-7786.MCR-16-0138

Kwon, Y. T., & Ciechanover, A. (2017). The Ubiquitin Code in the Ubiquitin-Proteasome System and Autophagy. *Trends in Biochemical Sciences*, *42*(11), 873–886. https://doi.org/10.1016/j.tibs.2017.09.002

Kwon, Y.-W., Kim, I.-J., Wu, D., Lu, J., Stock, W. A., Liu, Y., Huang, Y., Kang, H. C., DelRosario, R., Jen, K.-Y., Perez-Losada, J., Wei, G., Balmain, A., & Mao, J.-H. (2012). Pten regulates Aurora-A and cooperates with Fbxw7 in modulating radiation-induced tumor development. *Molecular Cancer Research: MCR*, *10*(6), 834–844. https://doi.org/10.1158/1541-7786.MCR-12-0025

Lake, M. W., Wuebbens, M. M., Rajagopalan, K. V., & Schindelin, H. (2001). Mechanism of ubiquitin activation revealed by the structure of a bacterial MoeB-MoaD complex. *Nature*, *414*(6861), 325–329. https://doi.org/10.1038/35104586

Lan, H., Tan, M., Zhang, Q., Yang, F., Wang, S., Li, H., Xiong, X., & Sun, Y. (2019). LSD1 destabilizes FBXW7 and abrogates FBXW7 functions independent of its demethylase activity. *Proceedings of the National Academy of Sciences*, *116*(25), 12311–12320. https://doi.org/10.1073/pnas.1902012116

Lechtenberg, B. C., Rajput, A., Sanishvili, R., Dobaczewska, M. K., Ware, C. F., Mace, P. D., & Riedl, S. J. (2016). Structure of a HOIP/E2~ubiquitin complex reveals RBR E3 ligase mechanism and regulation. *Nature*, *529*(7587), 546–550. https://doi.org/10.1038/nature16511

Lee, A. Y. L., Dubois, C. L., Sarai, K., Zarei, S., Schaeffer, D. F., Sander, M., & Kopp, J. L. (2019). Cell of origin affects tumour development and phenotype in pancreatic ductal adenocarcinoma. *Gut*, *68*(3), 487–498. https://doi.org/10.1136/GUTJNL-2017-314426

Lee, I., & Schindelin, H. (2008). Structural Insights into E1-Catalyzed Ubiquitin Activation and Transfer to Conjugating Enzymes. *Cell*, *134*(2), 268–278. https://doi.org/10.1016/j.cell.2008.05.046

Lee, J. W., Kang, H. S., Lee, J. Y., Lee, E. J., Rhim, H., Yoon, J. H., Seo, S. R., & Chung, K. C. (2012). The transcription factor STAT2 enhances proteasomal degradation of RCAN1 through the ubiquitin E3 ligase FBW7. *Biochemical and Biophysical Research Communications*, *420*(2), 404–410. https://doi.org/10.1016/j.bbrc.2012.03.007

Li, H., Wang, Z., Zhang, W., Qian, K., Xu, W., & Zhang, S. (2016). Fbxw7 regulates tumor apoptosis, growth arrest and the epithelial-to-mesenchymal transition in part through the RhoA signaling pathway in gastric cancer. *Cancer Letters*, *370*(1), 39–55. https://doi.org/10.1016/j.canlet.2015.10.006

Li, J., Pauley, A. M., Myers, R. L., Shuang, R., Brashler, J. R., Yan, R., Buhl, A. E., Ruble, C., & Gurney, M. E. (2002). SEL-10 interacts with presenilin 1, facilitates its ubiquitination, and alters A-beta peptide production. *Journal of Neurochemistry*, *82*(6), 1540–1548. https://doi.org/10.1046/j.1471-4159.2002.01105.x

Li, N., Babaei-Jadidi, R., Lorenzi, F., Spencer-Dene, B., Clarke, P., Domingo, E., Tulchinsky, E., Vries, R. G. J., Kerr, D., Pan, Y., He, Y., Bates, D. O., Tomlinson, I., Clevers, H., & Nateri, A. S. (2019). An FBXW7-ZEB2 axis links EMT and tumour microenvironment to promote colorectal cancer stem cells and chemoresistance. *Oncogenesis*, *8*(3), 1–17. https://doi.org/10.1038/s41389-019-0125-3

Li, Y., Hu, K., Xiao, X., Wu, W., Yan, H., Chen, H., Chen, Z., & Yin, D. (2018). FBW7 suppresses cell proliferation and G2/M cell cycle transition via promoting γ-catenin K63-linked ubiquitylation. *Biochemical and Biophysical Research Communications*, *497*(2), 473–479. https://doi.org/10.1016/j.bbrc.2018.01.192

Liao, S.-Y., Chiang, C.-W., Hsu, C.-H., Chen, Y.-T., Jen, J., Juan, H.-F., Lai, W.-W., & Wang, Y.-C. (2017). CK1δ/GSK3β/FBXW7α axis promotes degradation of the ZNF322A oncoprotein to suppress lung cancer progression. *Oncogene*, *36*(41), 5722–5733. https://doi.org/10.1038/onc.2017.168

Lin, H.-K., Chen, Z., Wang, G., Nardella, C., Lee, S.-W., Chan, C.-H., Chan, C.-H., Yang, W.-L., Wang, J., Egia, A., Nakayama, K. I., Cordon-Cardo, C., Teruya-Feldstein, J., & Pandolfi, P. P. (2010). Skp2 targeting suppresses tumorigenesis by Arf-p53-independent cellular senescence. *Nature*, *464*(7287), 374–379. https://doi.org/10.1038/nature08815

Lin, W. C., Rajbhandari, N., Liu, C., Sakamoto, K., Zhang, Q., Triplett, A. A., Batra, S. K., Opavsky, R., Felsher, D. W., DiMaio, D. J., Hollingsworth, M. A., Morris IV, J. P., Hebrok, M., Witkiewicz, A. K., Brody, J. R., Rui, H., & Wagner, K. U. (2013). Dormant cancer cells contribute to residual disease in a model of reversible pancreatic cancer. *Cancer Research*, *73*(6), 1821–1830. https://doi.org/10.1158/0008-5472.CAN-12-2067/658428/P/DORMANT-CANCER-CELLS-CONTRIBUTE-TO-RESIDUAL

Liu, N., Li, H., Li, S., Shen, M., Xiao, N., Chen, Y., Wang, Y., Wang, W., Wang, R., Wang, Q., Sun, J., & Wang, P. (2010). The Fbw7/human CDC4 tumor suppressor targets proproliferative factor KLF5 for ubiquitination and degradation through multiple phosphodegron motifs. *The Journal of Biological Chemistry*, *285*(24), 18858–18867. https://doi.org/10.1074/jbc.M109.099440

Liu, W., Tang, X., Qi, X., Fu, X., Ghimire, S., Ma, R., Li, S., Zhang, N., & Si, H. (2020). The Ubiquitin Conjugating Enzyme: An Important Ubiquitin Transfer Platform in Ubiquitin-Proteasome System. *International Journal of Molecular Sciences*, *21*(8), 2894. https://doi.org/10.3390/ijms21082894

Liu, Y., Huang, Y., Wang, Z., Huang, Y., Li, X., Louie, A., Wei, G., & Mao, J.-H. (2013). Temporal mTOR inhibition protects Fbxw7-deficient mice from radiation-induced tumor development. *Aging*, *5*(2), 111–119. https://doi.org/10.18632/aging.100535

Lochab, S., Pal, P., Kapoor, I., Kanaujiya, J. K., Sanyal, S., Behre, G., & Trivedi, A. K. (2013). E3 ubiquitin ligase Fbw7 negatively regulates granulocytic differentiation by targeting G-CSFR for degradation. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, *1833*(12), 2639–2652. https://doi.org/10.1016/j.bbamcr.2013.06.018

Loda, M., Cukor, B., Tam, S. W., Lavin, P., Fiorentino, M., Draetta, G. F., Jessup, J. M., & Pagano, M. (1997). Increased proteasome-dependent degradation of the cyclin-dependent kinase inhibitor p27 in aggressive colorectal carcinomas. *Nature Medicine*, *3*(2), 231–234. https://doi.org/10.1038/nm0297-231

Lois, L. M., & Lima, C. D. (2005). Structures of the SUMO E1 provide mechanistic insights into SUMO activation and E2 recruitment to E1. *The EMBO Journal*, *24*(3), 439–451. https://doi.org/10.1038/sj.emboj.7600552

Louvi, A., & Artavanis-Tsakonas, S. (2012). Notch and disease: A growing field. *Seminars in Cell & Developmental Biology*, *23*(4), 473–480. https://doi.org/10.1016/j.semcdb.2012.02.005

Lv, X., Li, J., Zhang, C., Hu, T., Li, S., He, S., Yan, H., Tan, Y., Lei, M., Wen, M., & Zuo, J. (2017). The role of hypoxia-inducible factors in tumor angiogenesis and cell metabolism. *Genes & Diseases*, *4*(1), 19–24. https://doi.org/10.1016/j.gendis.2016.11.003

Lv, X.-B., Wu, W., Tang, X., Wu, Y., Zhu, Y., Liu, Y., Cui, X., Chu, J., Hu, P., Li, J., Guo, Q., Cai, Z., Wu, J., Hu, K., & Ouyang, N. (2015). Regulation of SOX10 stability via ubiquitination-mediated degradation by Fbxw7α modulates melanoma cell migration. *Oncotarget*, *6*(34), 36370–36382. https://doi.org/10.18632/oncotarget.5639

Maddipati, R., Norgard, R. J., Baslan, T., Rathi, K. S., Zhang, A., Saeid, A., Higashihara, T., Wu, F., Kumar, A., Annamalai, V., Bhattacharya, S., Raman, P., Adkisson, C. A., Pitarresi, J. R., Wengyn, M. D., Yamazoe, T., Li, J., Balli, D., Lariviere, M. J., … Stanger, B. Z. (2022). MYC Levels Regulate Metastatic Heterogeneity in Pancreatic Adenocarcinoma. *Cancer Discovery*, *12*(2), 542–561. https://doi.org/10.1158/2159-8290.CD-20-1826/673841/AM/MYC-LEVELS-REGULATE-METASTATIC-HETEROGENEITY-IN

Makohon-Moore, A. P., Zhang, M., Reiter, J. G., Bozic, I., Allen, B., Kundu, D., Chatterjee, K., Wong, F., Jiao, Y., Kohutek, Z. A., Hong, J., McMahon, B., Wood, L. D., Hruban, R. H., Nowak, M. A., Papadopoulos, N., Kinzler, K. W., Vogelstein, B., & Iacobuzio-Donahue, C. A. (2016). Limited heterogeneity of known driver genes among the metastases of individual pancreatic cancer patients. *(Submitted)*, *49*(3), 358–366. https://doi.org/10.1038/ng.3764.Limited

Malyukova, A., Brown, S., Papa, R., O'Brien, R., Giles, J., Trahair, T. N., Dalla Pozza, L., Sutton, R., Liu, T., Haber, M., Norris, M. D., Lock, R. B., Sangfelt, O., & Marshall, G. M. (2013). FBXW7 regulates glucocorticoid response in T-cell acute lymphoblastic leukaemia by targeting the glucocorticoid receptor for degradation. *Leukemia*, *27*(5), 1053–1062. https://doi.org/10.1038/leu.2012.361

Manchado, E., Guillamot, M., de Cárcer, G., Eguren, M., Trickey, M., García-Higuera, I., Moreno, S., Yamano, H., Cañamero, M., & Malumbres, M. (2010). Targeting mitotic exit

leads to tumor regression in vivo: Modulation by Cdk1, Mastl, and the PP2A/B55α,δ phosphatase. *Cancer Cell*, *18*(6), 641–654. https://doi.org/10.1016/j.ccr.2010.10.028

Mantovani, F., Collavin, L., & Del Sal, G. (2019). Mutant p53 as a guardian of the cancer cell. *Cell Death and Differentiation*, *26*(2), 199–212. https://doi.org/10.1038/S41418-018-0246-9

Mao, J., Perez-losada, J., Wu, D., & Delrosario, R. (2004). Fbxw7/Cdc4 is a p53-dependent, haploinsufficient tumour suppressor gene. *Nature*, *432*, 775–779.

Mao, J.-H., Kim, I.-J., Wu, D., Climent, J., Kang, H. C., DelRosario, R., & Balmain, A. (2008). FBXW7 Targets mTOR for Degradation and Cooperates with PTEN in Tumor Suppression. *Science*, *321*(5895), 1499–1502. https://doi.org/10.1126/science.1162981

Maskey, D., Marlin, M. C., Kim, S., Kim, S., Ong, E.-C., Li, G., & Tsiokas, L. (2015). Cell cycle-dependent ubiquitylation and destruction of NDE1 by CDK5-FBW7 regulates ciliary length. *The EMBO Journal*, *34*(19), 2424–2440. https://doi.org/10.15252/embj.201490831

Maspero, E., Valentini, E., Mari, S., Cecatiello, V., Soffientini, P., Pasqualato, S., & Polo, S. (2013). Structure of a ubiquitin-loaded HECT ligase reveals the molecular basis for catalytic priming. *Nature Structural & Molecular Biology*, *20*(6), 696–701. https://doi.org/10.1038/nsmb.2566

McDonald, O. G., Li, X., Saunders, T., Tryggvadottir, R., Mentch, S. J., Warmoes, M. O., Word, A. E., Carrer, A., Salz, T. H., Natsume, S., Stauffer, K. M., Makohon-Moore, A., Zhong, Y., Wu, H., Wellen, K. E., Locasale, J. W., Iacobuzio-Donahue, C. A., & Feinberg, A. P. (2017). Epigenomic reprogramming during pancreatic cancer progression links anabolic glucose metabolism to distant metastasis. *Nature Genetics*, *49*(3), 367–376. https://doi.org/10.1038/ng.3753

Melo, S. A., Luecke, L. B., Kahlert, C., Fernandez, A. F., Gammon, S. T., Kaye, J., LeBleu, V. S., Mittendorf, E. A., Weitz, J., Rahbari, N., Reissfelder, C., Pilarsky, C., Fraga, M. F., Piwnica-Worms, D., & Kalluri, R. (2015). Glypican-1 identifies cancer exosomes and detects early pancreatic cancer. *Nature*, *523*(7559), 177–182. https://doi.org/10.1038/NATURE14581

Michels, J., O'Neill, J. W., Dallman, C. L., Mouzakiti, A., Habens, F., Brimmell, M., Zhang, K. Y. J., Craig, R. W., Marcusson, E. G., Johnson, P. W. M., & Packham, G. (2004). Mcl-1 is required for Akata6 B-lymphoma cell survival and is converted to a cell death molecule by efficient caspase-mediated cleavage. *Oncogene*, *23*(28), 4818–4827. https://doi.org/10.1038/sj.onc.1207648

Min, S.-H., Lau, A. W., Lee, T. H., Inuzuka, H., Wei, S., Huang, P., Shaik, S., Lee, D. Y., Finn, G., Balastik, M., Chen, C.-H., Luo, M., Tron, A. E., Decaprio, J. A., Zhou, X. Z., Wei, W., & Lu, K. P. (2012). Negative regulation of the stability and tumor suppressor function of Fbw7 by the Pin1 prolyl isomerase. *Molecular Cell*, *46*(6), 771–783. https://doi.org/10.1016/j.molcel.2012.04.012

Minella, A. C., Grim, J. E., Welcker, M., & Clurman, B. E. (2007). P53 and SCFFbw7 cooperatively restrain cyclin E-associated genome instability. *Oncogene*, *26*(48), 6948–6953. https://doi.org/10.1038/sj.onc.1210518

Minella, A. C., Welcker, M., & Clurman, B. E. (2005). Ras activity regulates cyclin E degradation by the Fbw7 pathway. *Proceedings of the National Academy of Sciences of the United States of America*, *102*(27), 9649–9654. https://doi.org/10.1073/pnas.0503677102

Miquel, M., Zhang, S., & Pilarsky, C. (2021). Pre-clinical Models of Metastasis in Pancreatic Cancer. *Frontiers in Cell and Developmental Biology*, *9*, 2825. https://doi.org/10.3389/FCELL.2021.748631/BIBTEX

Mirus, J. E., Zhang, Y., Li, C. I., Lokshin, A. E., Prentice, R. L., Hingorani, S. R., & Lampe, P. D. (2015). Cross-species antibody microarray interrogation identifies a 3-protein panel of plasma biomarkers for early diagnosis of pancreas cancer. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research*, *21*(7), 1764–1771. https://doi.org/10.1158/1078-0432.CCR-13-3474

Mishra, A., Emamgholi, F., Erlangga, Z., Hartleben, B., Unger, K., Wolff, K., Teichmann, U., Kessel, M., Woller, N., Kühnel, F., Dow, L. E., Manns, M. P., Vogel, A., Lowe, S. W., Saborowski, A., & Saborowski, M. (2020). Generation of focal mutations and large genomic deletions in the pancreas using inducible in vivo genome editing. *Carcinogenesis*, *41*(3), 334–344. https://doi.org/10.1093/CARCIN/BGZ108

Morton, J. P., Timpson, P., Karim, S. A., Ridgway, R. A., Athineos, D., Doyle, B., Jamieson, N. B., Oien, K. A., Lowy, A. M., Brunton, V. G., Frame, M. C., Evans, T. R. J., & Sansom, O. J. (2010). Mutant p53 drives metastasis and overcomes growth arrest/senescence in pancreatic cancer. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(1), 246–251. https://doi.org/10.1073/PNAS.0908428107

Nakajima, T., Kitagawa, K., Ohhata, T., Sakai, S., Uchida, C., Shibata, K., Minegishi, N., Yumimoto, K., Nakayama, K. I., Masumoto, K., Katou, F., Niida, H., & Kitagawa, M. (2015). Regulation of GATA-binding Protein 2 Levels via Ubiquitin-dependent Degradation by Fbw7. *The Journal of Biological Chemistry*, *290*(16), 10368–10381. https://doi.org/10.1074/jbc.M114.613018

Nakayama, S., Yumimoto, K., Kawamura, A., & Nakayama, K. I. (2018). Degradation of the endoplasmic reticulum–anchored transcription factor MyRF by the ubiquitin ligase SCFFbxw7 in a manner dependent on the kinase GSK-3. *Journal of Biological Chemistry*, *293*(15), 5705–5714. https://doi.org/10.1074/jbc.RA117.000741

Nemergut, M. E., Mizzen, C. A., Stukenberg, T., Allis, C. D., & Macara, I. G. (2001). Chromatin Docking and Exchange Activity Enhancement of RCC1 by Histones H2A and H2B. *Science*, *292*(5521), 1540–1543. https://doi.org/10.1126/science.292.5521.1540

Notta, F., Hahn, S. A., & Real, F. X. (2017). A genetic roadmap of pancreatic cancer: Still evolving. *Gut*, *66*(12), 2170–2178. https://doi.org/10.1136/GUTJNL-2016-313317

Offield, M. F., Jetton, T. L., Labosky, P. A., Ray, M., Stein, R. W., Magnuson, M. A., Hogan, B. L., & Wright, C. V. (1996). PDX-1 is required for pancreatic outgrowth and differentiation of the rostral duodenum. *Development (Cambridge, England)*, *122*(3), 983–995. https://doi.org/10.1242/dev.122.3.983

Ogunjimi, A. A., Briant, D. J., Pece-Barbara, N., Le Roy, C., Di Guglielmo, G. M., Kavsak, P., Rasmussen, R. K., Seet, B. T., Sicheri, F., & Wrana, J. L. (2005). Regulation of Smurf2 ubiquitin ligase activity by anchoring the E2 to the HECT domain. *Molecular Cell*, *19*(3), 297–308. https://doi.org/10.1016/j.molcel.2005.06.028

Oki, S., Ohta, T., Shioi, G., Hatanaka, H., Ogasawara, O., Okuda, Y., Kawaji, H., Nakaki, R., Sese, J., & Meno, C. (2018). ChIP-Atlas: A data-mining suite powered by full integration of public ChIP-seq data. *EMBO Reports*, *19*(12), e46255. https://doi.org/10.15252/embr.201846255

Olivier, M., Hollstein, M., & Hainaut, P. (2010). TP53 mutations in human cancers: Origins, consequences, and clinical use. *Cold Spring Harbor Perspectives in Biology*, *2*(1). https://doi.org/10.1101/CSHPERSPECT.A001008

O'Neil, J., Grim, J., Strack, P., Rao, S., Tibbitts, D., Winter, C., Hardwick, J., Welcker, M., Meijerink, J. P., Pieters, R., Draetta, G., Sears, R., Clurman, B. E., & Look, A. T. (2007). FBW7 mutations in leukemic cells mediate NOTCH pathway activation and resistance to γ-

secretase inhibitors. *Journal of Experimental Medicine*, *204*(8), 1813–1824. https://doi.org/10.1084/jem.20070876

Onoyama, I., Suzuki, A., Matsumoto, A., Tomita, K., Katagiri, H., Oike, Y., Nakayama, K., & Nakayama, K. I. (2011). Fbxw7 regulates lipid metabolism and cell fate decisions in the mouse liver. *The Journal of Clinical Investigation*, *121*(1), 342–354. https://doi.org/10.1172/JCI40725

Orlicky, S., Tang, X., Neduva, V., Elowe, N., Brown, E. D., Sicheri, F., & Tyers, M. (2010). An allosteric inhibitor of substrate recognition by the SCFCdc4 ubiquitin ligase. *Nature Biotechnology*, *28*(7), 733–737. https://doi.org/10.1038/nbt.1646

Orlicky, S., Tang, X., Willems, A., Tyers, M., & Sicheri, F. (2003). Structural basis for phosphodependent substrate selection and orientation by the SCFCdc4 ubiquitin ligase. *Cell*, *112*(2), 243–256. https://doi.org/10.1016/s0092-8674(03)00034-5

Otto, T., Horn, S., Brockmann, M., Eilers, U., Schüttrumpf, L., Popov, N., Kenney, A. M., Schulte, J. H., Beijersbergen, R., Christiansen, H., Berwanger, B., & Eilers, M. (2009). Stabilization of N-Myc is a critical function of Aurora A in human neuroblastoma. *Cancer Cell*, *15*(1), 67–78. https://doi.org/10.1016/j.ccr.2008.12.005

Ozkan, E., Yu, H., & Deisenhofer, J. (2005). Mechanistic insight into the allosteric activation of a ubiquitin-conjugating enzyme by RING-type ubiquitin ligases. *Proceedings of the National Academy of Sciences of the United States of America*, *102*(52), 18890–18895. https://doi.org/10.1073/pnas.0509418102

Pasca di Magliano, M., Biankin, A. V., Heiser, P. W., Cano, D. A., Gutierrez, P. J. A., Deramaudt, T., Segara, D., Dawson, A. C., Kench, J. G., Henshall, S. M., Sutherland, R. L., Dlugosz, A., Rustgi, A. K., & Hebrok, M. (2007). Common activation of canonical Wnt signaling in pancreatic adenocarcinoma. *PloS One*, *2*(11). https://doi.org/10.1371/JOURNAL.PONE.0001155

Patra, K. C., Kato, Y., Mizukami, Y., Widholz, S., Boukhali, M., Revenco, I., Grossman, E. A., Ji, F., Sadreyev, R. I., Liss, A. S., Screaton, R. A., Sakamoto, K., Ryan, D. P., Mino-Kenudson, M., Castillo, C. F. D., Nomura, D. K., Haas, W., & Bardeesy, N. (2018). Mutant GNAS drives pancreatic tumourigenesis by inducing PKA-mediated SIK suppression and reprogramming lipid metabolism. *Nature Cell Biology 2018 20:7*, *20*(7), 811–822. https://doi.org/10.1038/s41556-018-0122-3

Payne, S. N., Maher, M. E., Tran, N. H., Van De Hey, D. R., Foley, T. M., Yueh, A. E., Leystra, A. A., Pasch, C. A., Jeffrey, J. J., Clipson, L., Matkowskyj, K. A., & Deming, D. A. (2015). PIK3CA mutations can initiate pancreatic tumorigenesis and are targetable with PI3K inhibitors. *Oncogenesis 2015 4:10*, *4*(10), e169–e169. https://doi.org/10.1038/oncsis.2015.28

Pérez-Benavente, B., García, J. L., Rodríguez, M. S., Pineda-Lucena, A., Piechaczyk, M., Font de Mora, J., & Farràs, R. (2013). GSK3-SCFFBXW7 targets JunB for degradation in G2 to preserve chromatid cohesion before anaphase. *Oncogene*, *32*(17), 2189–2199. https://doi.org/10.1038/onc.2012.235

Peschiaroli, A., Dorrello, N. V., Guardavaccaro, D., Venere, M., Halazonetis, T., Sherman, N. E., & Pagano, M. (2006). SCFbetaTrCP-mediated degradation of Claspin regulates recovery from the DNA replication checkpoint response. *Molecular Cell*, *23*(3), 319–329. https://doi.org/10.1016/j.molcel.2006.06.013

Petroski, M. D., & Deshaies, R. J. (2005). Mechanism of lysine 48-linked ubiquitin-chain synthesis by the cullin-RING ubiquitin-ligase complex SCF-Cdc34. *Cell*, *123*(6), 1107–1120. https://doi.org/10.1016/j.cell.2005.09.033

Poole, B., Ohkuma, S., & Warburton, M. J. (1977). The accumulation of weakly basic substances in lysosomes and the inhibition of intracellular protein degradation. *Acta Biologica Et Medica Germanica*, *36*(11–12), 1777–1788.

Poole, B., Ohkuma, S., & Warburton, M. J. (1978). *Protein Turnover and Lysosome Function* (1st edition). Academic Press, New York. https://www.google.com/search?client=safari&rls=en&q=Poole%2C+B.%2C+Ohkuma%2C+S.+%26+Warburton%2C+M.+J.+in+Protein+Turnover+and+Lysosome+Function+(Segal%2C+H.+L.+%26+Doyle%2C+D.+J.%2C+eds)+43%E2%80%9358+(Academic+Press%2C+New+York%2C+1978).&ie=UTF-8&oe=UTF-8

Porta, M., Fabregat, X., Malats, N., Guarner, L., Carrato, A., de Miguel, A., Ruiz, L., Jariod, M., Costafreda, S., Coll, S., Alguacil, J., Corominas, J. M., Solà, R., Salas, A., & Real, F. X. (2005). Exocrine pancreatic cancer: Symptoms at presentation and their relation to tumour site and stage. *Clinical and Translational Oncology*, *7*(5), 189–197. https://doi.org/10.1007/BF02712816

Preis, M., & Korc, M. (2011). Signaling pathways in pancreatic cancer. *Critical Reviews in Eukaryotic Gene Expression*, *21*(2), 115–129. https://doi.org/10.1615/CRITREVEUKARGENEEXPR.V21.I2.20

Pruneda, J. N., Littlefield, P. J., Soss, S. E., Nordquist, K. A., Chazin, W. J., Brzovic, P. S., & Klevit, R. E. (2012). Structure of an E3:E2~Ub complex reveals an allosteric mechanism shared among RING/U-box ligases. *Molecular Cell*, *47*(6), 933–942. https://doi.org/10.1016/j.molcel.2012.07.001

Rabinovitz, M., & Fisher, J. M. (1964). Characteristics of the inhibition of hemoglobin synthesis in rabbit reticulocytes by threo-α-amino-β-chlorobutyric acid. *Biochimica et Biophysica Acta (BBA) - Specialized Section on Nucleic Acids and Related Subjects*, *91*(2), 313–322. https://doi.org/10.1016/0926-6550(64)90255-5

Rajagopalan, H., Jallepalli, P. V., Rago, C., Velculescu, V. E., Kinzler, K. W., Vogelstein, B., & Lengauer, C. (2004). Inactivation of hCDC4 can cause chromosomal instability. *Nature*, *428*(6978), 77–81. https://doi.org/10.1038/nature02313

Rajbhandari, N., Lin, W. chi, Wehde, B. L., Triplett, A. A., & Wagner, K. U. (2017). Autocrine IGF1 Signaling Mediates Pancreatic Tumor Cell Dormancy in the Absence of Oncogenic Drivers. *Cell Reports*, *18*(9), 2243–2255. https://doi.org/10.1016/J.CELREP.2017.02.013/ATTACHMENT/83E17AC9-D4E7-40DE-BBC0-60ECA133AD72/MMC1.PDF

Reavie, L., Buckley, S. M., Loizou, E., Takeishi, S., Aranda-Orgilles, B., Ndiaye-Lobry, D., Abdel-Wahab, O., Ibrahim, S., Nakayama, K. I., & Aifantis, I. (2013). Regulation of c-Myc ubiquitination controls chronic myelogenous leukemia initiation and progression. *Cancer Cell*, *23*(3), 362–375. https://doi.org/10.1016/j.ccr.2013.01.025

Rizo, J., & Südhof, T. C. (1998). C2-domains, Structure and Function of a Universal Ca2+-binding Domain*. *Journal of Biological Chemistry*, *273*(26), 15879–15882. https://doi.org/10.1074/jbc.273.26.15879

Roe, J., Hwang, C., Somerville, T. D. D., Park, Y., Tuveson, D. A., Vakoc, C. R., Young, C. M., Miyabayashi, K., Filippini, D., Creighton, B., Maiorino, L., Burkhart, R. A., Buscaglia, J. M., Kim, E. J., Grem, J. L., Lazenby, A. J., Grunkemeyer, J. A., Hollingsworth, M. A., Grandgenett, P. M., … Park, Y. (2017). *Enhancer Reprogramming Promotes*. 875–888. https://doi.org/10.1016/j.cell.2017.07.007

Rotin, D., & Kumar, S. (2009). Physiological functions of the HECT family of ubiquitin ligases. *Nature Reviews. Molecular Cell Biology*, *10*(6), 398–409. https://doi.org/10.1038/nrm2690

Ryan, D. P., Hong, T. S., & Bardeesy, N. (2014). Pancreatic Adenocarcinoma. *New England Journal of Medicine*, *371*(11), 1039–1049. https://doi.org/10.1056/NEJMra1404198

Sackton, K. L., Dimova, N., Zeng, X., Tian, W., Zhang, M., Sackton, T. B., Meaders, J., Pfaff, K. L., Sigoillot, F., Yu, H., Luo, X., & King, R. W. (2014). Synergistic blockade of mitotic exit by two chemical inhibitors of the APC/C. *Nature*, *514*(7524), 646–649. https://doi.org/10.1038/nature13660

Saiki, Y., Jiang, C., Ohmuraya, M., & Furukawa, T. (2022). Genetic Mutations of Pancreatic Cancer and Genetically Engineered Mouse Models. *Cancers*, *14*(1). https://doi.org/10.3390/CANCERS14010071

Sanborn, J. Z., Chung, J., Purdom, E., Wang, N. J., Kakavand, H., Wilmott, J. S., Butler, T., Thompson, J. F., Mann, G. J., Haydu, L. E., Saw, R. P. M., Busam, K. J., Lo, R. S., Collisson, E. A., Hur, J. S., Spellman, P. T., Cleaver, J. E., Gray, J. W., Huh, N., … Cho, R. J. (2015). Phylogenetic analyses of melanoma reveal complex patterns of metastatic dissemination. *Proceedings of the National Academy of Sciences of the United States of America*, *112*(35), 10995–11000. https://doi.org/10.1073/pnas.1508074112

Sanchez, D., & Cheung, W. L. (2015). Pathology of pancreatic tumors. *Translational Cancer Research*, *4*(6). https://doi.org/10.21037/5993

Sancho, R., Blake, S. M., Tendeng, C., Clurman, B. E., Lewis, J., & Behrens, A. (2013). Fbw7 Repression by Hes5 Creates a Feedback Loop That Modulates Notch-Mediated Intestinal and Neural Stem Cell Fate Decisions. *PLOS Biology*, *11*(6), e1001586. https://doi.org/10.1371/journal.pbio.1001586

Sancho, R., Gruber, R., Gu, G., & Behrens, A. (2014a). Loss of Fbw7 Reprograms Adult Pancreatic Ductal Cells into α, δ, and β Cells. *Cell Stem Cell*, *15*(2), 139–153. https://doi.org/10.1016/j.stem.2014.06.019

Sancho, R., Gruber, R., Gu, G., & Behrens, A. (2014b). Loss of Fbw7 Reprograms Adult Pancreatic Ductal Cells into α, δ, and β Cells. *Cell Stem Cell*, *15*(2), 139–153. https://doi.org/10.1016/j.stem.2014.06.019

Sandgren, E. P., Quaife, C. J., Paulovich, A. G., Palmiter, R. D., & Brinster, R. L. (1991). Pancreatic tumor pathogenesis reflects the causative genetic lesion. *Proceedings of the National Academy of Sciences*, *88*(1), 93–97. https://doi.org/10.1073/pnas.88.1.93

Sato, T., Vries, R. G., Snippert, H. J., Van De Wetering, M., Barker, N., Stange, D. E., Van Es, J. H., Abo, A., Kujala, P., Peters, P. J., & Clevers, H. (2009). Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. *Nature*, *459*(7244), 262–265. https://doi.org/10.1038/NATURE07935

Sausen, M., Phallen, J., Adleff, V., Jones, S., Leary, R. J., Barrett, M. T., Anagnostou, V., Parpart-Li, S., Murphy, D., Li, Q. K., Hruban, C. A., Scharpf, R., White, J. R., O'Dwyer, P. J., Allen, P. J., Eshleman, J. R., Thompson, C. B., Klimstra, D. S., Linehan, D. C., … Velculescu, V. E. (2015). Clinical implications of genomic alterations in the tumour and circulation of pancreatic cancer patients. *Nature Communications 2015 6:1*, *6*(1), 1–6. https://doi.org/10.1038/ncomms8686

Savage, K. I., & Harkin, D. P. (2015). BRCA1, a "complex" protein involved in the maintenance of genomic stability. *The FEBS Journal*, *282*(4), 630–646. https://doi.org/10.1111/febs.13150

Schimke, R. T. (1976). Protein degradation in vivo and its regulation. *Circulation Research*, *38*(5 Suppl 1), I131-137.

Schoenheimer, R. (1942). *The dynamic state of body constituents*. Harvard University Press. https://wellcomecollection.org/works/ac5fuk72/items

Schönhuber, N., Seidler, B., Schuck, K., Veltkamp, C., Schachtler, C., Zukowska, M., Eser, S., Feyerabend, T. B., Paul, M. C., Eser, P., Klein, S., Lowy, A. M., Banerjee, R., Yang, F., Lee, C. L., Moding, E. J., Kirsch, D. G., Scheideler, A., Alessi, D. R., … Saur, D. (2014). A next-generation dual-recombinase system for time- and host-specific targeting of pancreatic cancer. *Nature Medicine 2014 20:11*, *20*(11), 1340–1347. https://doi.org/10.1038/nm.3646

SEER Cancer Stat Facts: Pancreatic Cancer. National Cancer Institute. Bethesda, MD. (n.d.). Retrieved June 22, 2022, from https://seer.cancer.gov/statfacts/html/pancreas.html

Shim, E.-H., Johnson, L., Noh, H.-L., Kim, Y.-J., Sun, H., Zeiss, C., & Zhang, H. (2003). Expression of the F-box protein SKP2 induces hyperplasia, dysplasia, and low-grade carcinoma in the mouse prostate. *Cancer Research*, *63*(7), 1583–1588.

Simpson, M. V. (1953). The release of labeled amino acids from the proteins of rat liver slices. *The Journal of Biological Chemistry*, *201*(1), 143–154.

Singh, K., Pruski, M., Bland, R., Younes, M., Guha, S., Thosani, N., Maitra, A., Cash, B. D., McAllister, F., Logsdon, C. D., Chang, J. T., & Bailey-Lundberg, J. M. (2020). Kras mutation rate precisely orchestrates ductal derived pancreatic intraepithelial neoplasia and pancreatic cancer. *Laboratory Investigation 2020 101:2*, *101*(2), 177–192. https://doi.org/10.1038/s41374-020-00490-5

Skaar, J. R., Pagan, J. K., & Pagano, M. (2014). SCF ubiquitin ligase-targeted therapies. *Nature Reviews. Drug Discovery*, *13*(12), 889–903. https://doi.org/10.1038/nrd4432

Smith, E., & Cochrane, W. J. (1946). CYSTIC ORGANOID TERATOMA: (Report of a Case). *Canadian Medical Association Journal*, *55*(2), 151–152.

Soheilifar, M. H., Masoudi-Khoram, N., Madadi, S., Nobari, S., Maadi, H., Keshmiri Neghab, H., Amini, R., & Pishnamazi, M. (2021). Angioregulatory microRNAs in breast cancer: Molecular mechanistic basis and implications for therapeutic strategies. *Journal of Advanced Research*, *37*, 235–253. https://doi.org/10.1016/j.jare.2021.06.019

Song, Y., Lai, L., Chong, Z., He, J., Zhang, Y., Xue, Y., Xie, Y., Chen, S., Dong, P., Chen, L., Chen, Z., Dai, F., Wan, X., Xiao, P., Cao, X., Liu, Y., & Wang, Q. (2017). E3 ligase FBXW7 is critical for RIG-I stabilization during antiviral responses. *Nature Communications*, *8*(1), 14654. https://doi.org/10.1038/ncomms14654

Spruck, C. H., Strohmaier, H., Sangfelt, O., Müller, H. M., Hubalek, M., Müller-Holzner, E., Marth, C., Widschwendter, M., & Reed, S. I. (2002). HCDC4 gene mutations in endometrial cancer. *Cancer Research*, *62*(16), 4535–4539.

Stewart, M. D., Ritterhoff, T., Klevit, R. E., & Brzovic, P. S. (2016). E2 enzymes: More than just middle men. *Cell Research*, *26*(4), 423–440. https://doi.org/10.1038/cr.2016.35

Strohmaier, H., Spruck, C. H., Kaiser, P., Won, K. A., Sangfelt, O., & Reed, S. I. (2001). Human F-box protein hCdc4 targets cyclin E for proteolysis and is mutated in a breast cancer cell line. *Nature*, *413*(6853), 316–322. https://doi.org/10.1038/35095076

Sugiyama, S., Yumimoto, K., Inoue, I., & Nakayama, K. I. (2019). SCFFbxw7 ubiquitinates KLF7 for degradation in a manner dependent on GSK-3-mediated phosphorylation. *Genes to Cells*, *24*(5), 354–365. https://doi.org/10.1111/gtc.12680

Sun, Y., & Li, X. (2014). The Canonical Wnt Signal Restricts the Glycogen Synthase Kinase 3/Fbw7-Dependent Ubiquitination and Degradation of Eya1 Phosphatase. *Molecular and Cellular Biology*, *34*(13), 2409–2417. https://doi.org/10.1128/MCB.00104-14

Sundqvist, A., Bengoechea-Alonso, M. T., Ye, X., Lukiyanchuk, V., Jin, J., Harper, J. W., & Ericsson, J. (2005). Control of lipid metabolism by phosphorylation-dependent degradation of the SREBP family of transcription factors by SCFFbw7. *Cell Metabolism*, *1*(6), 379–391. https://doi.org/10.1016/j.cmet.2005.04.010

Szczepanowski, R. H., Filipek, R., & Bochtler, M. (2005). Crystal structure of a fragment of mouse ubiquitin-activating enzyme. *The Journal of Biological Chemistry*, *280*(23), 22006–22011. https://doi.org/10.1074/jbc.M502583200

Takada, M., Zhuang, M., Inuzuka, H., Zhang, J., Zurlo, G., Zhang, J., & Zhang, Q. (2016). EglN2 contributes to triple negative breast tumorigenesis by functioning as a substrate for the FBW7 tumor suppressor. *Oncotarget*, *8*(4), 6787–6795. https://doi.org/10.18632/oncotarget.14290

Takahashi, T. S., Hirade, Y., Toma, A., Sato, Y., Yamagata, A., Goto-Ito, S., Tomita, A., Nakada, S., & Fukai, S. (2018). Structural insights into two distinct binding modules for Lys63-linked polyubiquitin chains in RNF168. *Nature Communications*, *9*(1), 170. https://doi.org/10.1038/s41467-017-02345-y

Taki, K., Ohmuraya, M., Tanji, E., Komatsu, H., Hashimoto, D., Semba, K., Araki, K., Kawaguchi, Y., Baba, H., & Furukawa, T. (2015). GNASR201H and KrasG12D cooperate to promote murine pancreatic tumorigenesis recapitulating human intraductal papillary mucinous neoplasm. *Oncogene 2016 35:18*, *35*(18), 2407–2412. https://doi.org/10.1038/onc.2015.294

Tanaka, K., Waxman, L., & Goldberg, A. L. (1983). ATP serves two distinct roles in protein degradation in reticulocytes, one requiring and one independent of ubiquitin. *Journal of Cell Biology*, *96*(6), 1580–1585. https://doi.org/10.1083/jcb.96.6.1580

Teng, C.-L., Hsieh, Y.-C., Phan, L., Shin, J., Gully, C., Velazquez-Torres, G., Skerl, S., Yeung, S.-C. J., Hsu, S.-L., & Lee, M.-H. (2012). FBXW7 is involved in Aurora B degradation. *Cell Cycle*, *11*(21), 4059–4068. https://doi.org/10.4161/cc.22381

Thiery, J. P. (2002). Epithelial-mesenchymal transitions in tumour progression. *Nature Reviews. Cancer*, *2*(6), 442–454. https://doi.org/10.1038/nrc822

Thompson, B. J., Jankovic, V., Gao, J., Buonamici, S., Vest, A., Lee, J. M., Zavadil, J., Nimer, S. D., & Aifantis, I. (2008). Control of hematopoietic stem cell quiescence by the E3 ubiquitin ligase Fbw7. *The Journal of Experimental Medicine*, *205*(6), 1395–1408. https://doi.org/10.1084/jem.20080277

Tian, M., Bai, C., Lin, Q., Lin, H., Liu, M., Ding, F., & Wang, H.-R. (2011). Binding of RhoA by the C2 domain of E3 ligase Smurf1 is essential for Smurf1-regulated RhoA ubiquitination and cell protrusive activity. *FEBS Letters*, *585*(14), 2199–2204. https://doi.org/10.1016/j.febslet.2011.06.016

Tjarda Van Heek, N., Kuhlmann, K. F. D., Scholten, R. J., De Castro, S. M. M., Busch, O. R. C., Van Gulik, T. M., Obertop, H., Gouma, D. J., Ihse, Cameron, Russell, Nordlinger, Lerut, & Clavien. (2005). Hospital volume and mortality after pancreatic resection: A systematic review and an evaluation of intervention in the Netherlands. *Annals of Surgery*, *242*(6), 781–790. https://doi.org/10.1097/01.SLA.0000188462.00249.36

Tron, A. E., Arai, T., Duda, D. M., Kuwabara, H., Olszewski, J. L., Fujiwara, Y., Bahamon, B. N., Signoretti, S., Schulman, B. A., & DeCaprio, J. A. (2012). The glomuvenous malformation protein Glomulin binds Rbx1 and regulates cullin RING ligase-mediated turnover of Fbw7. *Molecular Cell*, *46*(1), 67–78. https://doi.org/10.1016/j.molcel.2012.02.005

Tu, K., Yang, W., Li, C., Zheng, X., Lu, Z., Guo, C., Yao, Y., & Liu, Q. (2014). Fbxw7 is an independent prognostic marker and induces apoptosis and growth arrest by regulating YAP abundance in hepatocellular carcinoma. *Molecular Cancer*, *13*, 110. https://doi.org/10.1186/1476-4598-13-110

van Wijk, S. J. L., & Timmers, H. T. M. (2010). The family of ubiquitin-conjugating enzymes (E2s): Deciding between life and death of proteins. *The FASEB Journal*, *24*(4), 981–993. https://doi.org/10.1096/fj.09-136259

Verdecia, M. A., Joazeiro, C. A. P., Wells, N. J., Ferrer, J.-L., Bowman, M. E., Hunter, T., & Noel, J. P. (2003). Conformational flexibility underlies ubiquitin ligation mediated by the WWP1 HECT domain E3 ligase. *Molecular Cell*, *11*(1), 249–259. https://doi.org/10.1016/s1097-2765(02)00774-8

Von Figura, G., Fukuda, A., Roy, N., Liku, M. E., Morris Iv, J. P., Kim, G. E., Russ, H. A., Firpo, M. A., Mulvihill, S. J., Dawson, D. W., Ferrer, J., Mueller, W. F., Busch, A., Hertel, K. J., & Hebrok, M. (2014). The chromatin regulator Brg1 suppresses formation of intraductal papillary mucinous neoplasm and pancreatic ductal adenocarcinoma. *Nature Cell Biology 2014 16:3*, *16*(3), 255–267. https://doi.org/10.1038/ncb2916

Wade, M., Li, Y.-C., & Wahl, G. M. (2013). MDM2, MDMX and p53 in oncogenesis and cancer therapy. *Nature Reviews. Cancer*, *13*(2), 83–96. https://doi.org/10.1038/nrc3430

Walden, H., Podgorski, M. S., & Schulman, B. A. (2003). Insights into the ubiquitin transfer cascade from the structure of the activating enzyme for NEDD8. *Nature*, *422*(6929), 330–334. https://doi.org/10.1038/nature01456

Wang, J., Jo, U., Joo, S. Y., & Kim, H. (2016). FBW7 regulates DNA interstrand cross-link repair by modulating FAAP20 degradation. *Oncotarget*, *7*(24), 35724–35740. https://doi.org/10.18632/oncotarget.9595

Wang, R., Wang, Y., Liu, N., Ren, C., Jiang, C., Zhang, K., Yu, S., Chen, Y., Tang, H., Deng, Q., Fu, C., Wang, Y., Li, R., Liu, M., Pan, W., & Wang, P. (2013). FBW7 regulates endothelial functions by targeting KLF2 for ubiquitination and degradation. *Cell Research*, *23*(6), 803–819. https://doi.org/10.1038/cr.2013.42

Wang, W., Friedland, S. C., Guo, B., O'Dell, M. R., Alexander, W. B., Whitney-Miller, C. L., Agostini-Vulaj, D., Huber, A. R., Myers, J. R., Ashton, J. M., Dunne, R. F., Steiner, L. A., & Hezel, A. F. (2019). ARID1A, a SWI/SNF subunit, is critical to acinar cell homeostasis and regeneration and is a barrier to transformation and epithelial-mesenchymal transition in the pancreas. *Gut*, *68*(7), 1245–1258. https://doi.org/10.1136/GUTJNL-2017-315541

Wang, Z., Liu, Y., Zhang, P., Zhang, W., Wang, W., Curr, K., Wei, G., & Mao, J.-H. (2013). FAM83D promotes cell proliferation and motility by downregulating tumor suppressor gene FBXW7. *Oncotarget*, *4*(12), 2476–2486. https://doi.org/10.18632/oncotarget.1581

Watanabe, N., Arai, H., Nishihara, Y., Taniguchi, M., Watanabe, N., Hunter, T., & Osada, H. (2004). M-phase kinases induce phospho-dependent ubiquitination of somatic Wee1 by SCFbeta-TrCP. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(13), 4419–4424. https://doi.org/10.1073/pnas.0307700101

Waxman, L., Fagan, J. M., & Goldberg, A. L. (1987). Demonstration of two distinct high molecular weight proteases in rabbit reticulocytes, one of which degrades ubiquitin conjugates. *The Journal of Biological Chemistry*, *262*(6), 2451–2457.

Wei, W., Jin, J., Schlisio, S., Harper, J. W., & Kaelin, W. G. (2005). The v-Jun point mutation allows c-Jun to escape GSK3-dependent recognition and destruction by the Fbw7 ubiquitin ligase. *Cancer Cell*, *8*(1), 25–33. https://doi.org/10.1016/j.ccr.2005.06.005

Welcker, M., & Clurman, B. E. (2005). The SV40 large T antigen contains a decoy phosphodegron that mediates its interactions with Fbw7/hCdc4. *The Journal of Biological Chemistry*, *280*(9), 7654–7658. https://doi.org/10.1074/jbc.M413377200

Welcker, M., & Clurman, B. E. (2008). FBW7 ubiquitin ligase: A tumour suppressor at the crossroads of cell division, growth and differentiation. *Nature Reviews Cancer*, *8*(2), 83–93. https://doi.org/10.1038/nrc2290

Welcker, M., Larimore, E. A., Frappier, L., & Clurman, B. E. (2011). Nucleolar targeting of the fbw7 ubiquitin ligase by a pseudosubstrate and glycogen synthase kinase 3. *Molecular and Cellular Biology*, *31*(6), 1214–1224. https://doi.org/10.1128/MCB.01347-10

Welcker, M., Larimore, E. A., Swanger, J., Bengoechea-Alonso, M. T., Grim, J. E., Ericsson, J., Zheng, N., & Clurman, B. E. (2013). Fbw7 dimerization determines the specificity and robustness of substrate degradation. *Genes & Development*, *27*(23), 2531–2536. https://doi.org/10.1101/gad.229195.113

Welcker, M., Orian, A., Grim, J. E., Grim, J. A., Eisenman, R. N., & Clurman, B. E. (2004). A nucleolar isoform of the Fbw7 ubiquitin ligase regulates c-Myc and cell size. *Current Biology: CB*, *14*(20), 1852–1857. https://doi.org/10.1016/j.cub.2004.09.083

Welcker, M., Orian, A., Jin, J., Grim, J. E., Grim, J. A., Harper, J. W., Eisenman, R. N., & Clurman, B. E. (2004). The Fbw7 tumor suppressor regulates glycogen synthase kinase 3 phosphorylation-dependent c-Myc protein degradation. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(24), 9085–9090. https://doi.org/10.1073/pnas.0402770101

Welcker, M., Singer, J., Loeb, K. R., Grim, J., Bloecher, A., Gurien-West, M., Clurman, B. E., & Roberts, J. M. (2003). Multisite phosphorylation by Cdk2 and GSK3 controls cyclin E degradation. *Molecular Cell*, *12*(2), 381–392. https://doi.org/10.1016/s1097-2765(03)00287-9

Weng, A. P., Ferrando, A. A., Lee, W., Morris, J. P., Silverman, L. B., Sanchez-Irizarry, C., Blacklow, S. C., Look, A. T., & Aster, J. C. (2004). Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia. *Science (New York, N.Y.)*, *306*(5694), 269–271. https://doi.org/10.1126/science.1102160

Wenzel, D. M., Lissounov, A., Brzovic, P. S., & Klevit, R. E. (2011). UBCH7 reactivity profile reveals parkin and HHARI to be RING/HECT hybrids. *Nature*, *474*(7349), 105–108. https://doi.org/10.1038/nature09966

Whittle, M. C., Izeradjene, K., Geetha Rani, P., Feng, L., Carlson, M. A., DelGiorno, K. E., Wood, L. D., Goggins, M., Hruban, R. H., Chang, A. E., Calses, P., Thorsen, S. M., & Hingorani, S. R. (2015). RUNX3 controls a metastatic switch in pancreatic ductal adenocarcinoma. *Cell*, *161*(6), 1345–1360. https://doi.org/10.1016/j.cell.2015.04.048

Wilkinson, K. D., Urban, M. K., & Haas, A. L. (1980). Ubiquitin is the ATP-dependent proteolysis factor I of rabbit reticulocytes. *The Journal of Biological Chemistry*, *255*(16), 7529–7532.

Winston, J. T., Koepp, D. M., Zhu, C., Elledge, S. J., & Harper, J. W. (1999). A family of mammalian F-box proteins. *Current Biology*, *9*(20), 1180–1182. https://doi.org/10.1016/S0960-9822(00)80021-4

Wrana, J. L. (2009). The secret life of Smad4. *Cell*, *136*(1), 13–14. https://doi.org/10.1016/J.CELL.2008.12.028

Wu, J., Liu, X., Nayak, S. G., Pitarresi, J. R., Cuitiño, M. C., Yu, L., Hildreth, B. E., Thies, K. A., Schilling, D. J., Fernandez, S. A., Leone, G., & Ostrowski, M. C. (2017). Generation of a

pancreatic cancer model using a Pdx1-Flp recombinase knock-in allele. *PLOS ONE*, *12*(9), e0184984. https://doi.org/10.1371/JOURNAL.PONE.0184984

Wu, R.-C., Feng, Q., Lonard, D. M., & O'Malley, B. W. (2007). SRC-3 Coactivator Functional Lifetime Is Regulated by a Phospho-Dependent Ubiquitin Time Clock. *Cell*, *129*(6), 1125–1140. https://doi.org/10.1016/j.cell.2007.04.039

Xie, C. M., Tan, M., Lin, X. T., Wu, D., Jiang, Y., Tan, Y., Li, H., Ma, Y., Xiong, X., & Sun, Y. (2019). The FBXW7-SHOC2-Raptor Axis Controls the Cross-Talks between the RAS-ERK and mTORC1 Signaling Pathways. *Cell Reports*, *26*(11), 3037-3050.e4. https://doi.org/10.1016/j.celrep.2019.02.052

Yachida, S., Jones, S., Bozic, I., Antal, T., Leary, R., Fu, B., Kamiyama, M., Hruban, R. H., Eshleman, J. R., Nowak, M. A., Velculescu, V. E., Kinzler, K. W., Vogelstein, B., & Iacobuzio-Donahue, C. A. (2010). Distant metastasis occurs late during the genetic evolution of pancreatic cancer. *Nature*, *467*(7319), 1114–1117. https://doi.org/10.1038/nature09515

Yada, M., Hatakeyama, S., Kamura, T., Nishiyama, M., Tsunematsu, R., Imaki, H., Ishida, N., Okumura, F., Nakayama, K., & Nakayama, K. I. (2004). Phosphorylation-dependent degradation of c-Myc is mediated by the F-box protein Fbw7. *The EMBO Journal*, *23*(10), 2116–2125. https://doi.org/10.1038/sj.emboj.7600217

Yalla, K., Elliott, C., Day, J. P., Findlay, J., Barratt, S., Hughes, Z. A., Wilson, L., Whiteley, E., Popiolek, M., Li, Y., Dunlop, J., Killick, R., Adams, D. R., Brandon, N. J., Houslay, M. D., Hao, B., & Baillie, G. S. (2018). FBXW7 regulates DISC1 stability via the ubiquitin-proteosome system. *Molecular Psychiatry*, *23*(5), 1278–1286. https://doi.org/10.1038/mp.2017.138

Yang, Q., Zhao, J., Chen, D., & Wang, Y. (2021). E3 ubiquitin ligases: Styles, structures and functions. *Molecular Biomedicine*, *2*(1), 23. https://doi.org/10.1186/s43556-021-00043-2

Yao, S., Xu, F., Chen, Y., Ge, Y., Zhang, F., Huang, H., Li, L., Lin, D., Luo, X., Xu, J., Luo, D., Zhu, X., & Liu, Y. (2017). Fbw7 regulates apoptosis in activated B-cell like diffuse large B-cell lymphoma by targeting Stat3 for ubiquitylation and degradation. *Journal of Experimental & Clinical Cancer Research : CR*, *36*, 10. https://doi.org/10.1186/s13046-016-0476-y

Yau, R., & Rape, M. (2016). The increasing complexity of the ubiquitin code. *Nature Cell Biology*, *18*(6), 579–586. https://doi.org/10.1038/ncb3358

Ye, Z., Zhuo, Q., Hu, Q., Xu, X., Mengqi liu, Zhang, Z., Xu, W., Liu, W., Fan, G., Qin, Y., Yu, X., & Ji, S. (2021). FBW7-NRA41-SCD1 axis synchronously regulates apoptosis and ferroptosis in pancreatic cancer cells. *Redox Biology*, *38*, 101807. https://doi.org/10.1016/j.redox.2020.101807

Yeh, C.-H., Bellon, M., Pancewicz-Wojtkiewicz, J., & Nicot, C. (2016). Oncogenic mutations in the FBXW7 gene of adult T-cell leukemia patients. *Proceedings of the National Academy of Sciences of the United States of America*, *113*(24), 6731–6736. https://doi.org/10.1073/pnas.1601537113

Yu, S., Wang, F., Tan, X., Gao, G., Pan, W.-J., Luan, Y., & Ge, X. (2018). FBW7 targets KLF10 for ubiquitin-dependent degradation. *Biochemical and Biophysical Research Communications*, *495*(2), 2092–2097. https://doi.org/10.1016/j.bbrc.2017.11.187

Yu, Y., Yang, G., Huang, H., Fu, Z., Cao, Z., Zheng, L., You, L., & Zhang, T. (2021). Preclinical models of pancreatic ductal adenocarcinoma: Challenges and opportunities in the era of precision medicine. *Journal of Experimental & Clinical Cancer Research 2021 40:1*, *40*(1), 1–13. https://doi.org/10.1186/S13046-020-01787-5

Yumimoto, K., Matsumoto, M., Onoyama, I., Imaizumi, K., & Nakayama, K. I. (2013). F-box and WD Repeat Domain-containing-7 (Fbxw7) Protein Targets Endoplasmic Reticulum-anchored Osteogenic and Chondrogenic Transcriptional Factors for Degradation. *The Journal of Biological Chemistry*, *288*(40), 28488–28502. https://doi.org/10.1074/jbc.M113.465179

Yumimoto, K., & Nakayama, K. I. (n.d.). *Recent insight into the role of FBXW7 as a tumor suppressor—ClinicalKey*. Retrieved August 17, 2022, from https://www-clinicalkey-com.ezp-prod1.hul.harvard.edu/#!/content/playContent/1-s2.0-S1044579X2030050X?scrollTo=%23bib0030

Zeng, T., Wang, Q., Fu, J., Lin, Q., Bi, J., Ding, W., Qiao, Y., Zhang, S., Zhao, W., Lin, H., Wang, M., Lu, B., Deng, X., Zhou, D., Yin, Z., & Wang, H.-R. (2014). Impeded Nedd4-1-mediated Ras degradation underlies Ras-driven tumorigenesis. *Cell Reports*, *7*(3), 871–882. https://doi.org/10.1016/j.celrep.2014.03.045

Zeng, X., Sigoillot, F., Gaur, S., Choi, S., Pfaff, K. L., Oh, D.-C., Hathaway, N., Dimova, N., Cuny, G. D., & King, R. W. (2010). Pharmacologic inhibition of the anaphase-promoting complex induces a spindle checkpoint-dependent mitotic arrest in the absence of spindle damage. *Cancer Cell*, *18*(4), 382–395. https://doi.org/10.1016/j.ccr.2010.08.010

Zhan, P., Wang, Y., Zhao, S., Liu, C., Wang, Y., Wen, M., Mao, J.-H., Wei, G., & Zhang, P. (2015). FBXW7 negatively regulates ENO1 expression and function in colorectal cancer. *Laboratory Investigation*, *95*(9), 995–1004. https://doi.org/10.1038/labinvest.2015.71

Zhang, B., Golding, B. T., & Hardcastle, I. R. (2015). Small-molecule MDM2-p53 inhibitors: Recent advances. *Future Medicinal Chemistry*, *7*(5), 631–645. https://doi.org/10.4155/fmc.15.13

Zhang, C., & Clarke, P. R. (2000). Chromatin-Independent Nuclear Envelope Assembly Induced by Ran GTPase in Xenopus Egg Extracts. *Science*, *288*(5470), 1429–1432. https://doi.org/10.1126/science.288.5470.1429

Zhang, P., Cao, L., Fan, P., Mei, Y., & Wu, M. (2016). LncRNA-MIF, a c-Myc-activated long non-coding RNA, suppresses glycolysis by promoting Fbxw7-mediated c-Myc degradation. *EMBO Reports*, *17*(8), 1204–1220. https://doi.org/10.15252/embr.201642067

Zhang, Q., Karnak, D., Tan, M., Lawrence, T. S., Morgan, M. A., & Sun, Y. (2016). FBXW7 Facilitates Nonhomologous End-Joining via K63-Linked Polyubiquitylation of XRCC4. *Molecular Cell*, *61*(3), 419–433. https://doi.org/10.1016/j.molcel.2015.12.010

Zhang, Q., Zhang, Y., Parsels, J. D., Lohse, I., Lawrence, T. S., Pasca di Magliano, M., Sun, Y., & Morgan, M. A. (2016). Fbxw7 Deletion Accelerates KrasG12D-Driven Pancreatic Tumorigenesis via Yap Accumulation. *Neoplasia (New York, N.Y.)*, *18*(11), 666–673. https://doi.org/10.1016/j.neo.2016.08.009

Zhang, S., Yong, L. K., Li, D., Cubas, R., Chen, C., & Yao, Q. (2013). Mesothelin virus-like particle immunization controls pancreatic cancer growth through CD8+ T cell induction and reduction in the frequency of CD4+ foxp3+ ICOS- regulatory T cells. *PloS One*, *8*(7). https://doi.org/10.1371/JOURNAL.PONE.0068303

Zhao, B., Tsai, Y. C., Jin, B., Wang, B., Wang, Y., Zhou, H., Carpenter, T., Weissman, A. M., & Yin, J. (2020). Protein Engineering in the Ubiquitin System: Tools for Discovery and Beyond. *Pharmacological Reviews*, *72*(2), 380–413. https://doi.org/10.1124/pr.118.015651

Zhao, E., Maj, T., Kryczek, I., Li, W., Wu, K., Zhao, L., Wei, S., Crespo, J., Wan, S., Vatan, L., Szeliga, W., Shao, I., Wang, Y., Liu, Y., Varambally, S., Chinnaiyan, A. M., Welling, T. H., Marquez, V., Kotarski, J., … Zou, W. (2016). Cancer mediates effector T cell dysfunction by

targeting microRNAs and EZH2 via glycolysis restriction. *Nature Immunology*, *17*(1), 95–103. https://doi.org/10.1038/ni.3313

Zhao, J., Xiong, X., Li, Y., Liu, X., Wang, T., Zhang, H., Jiao, Y., Jiang, J., Zhang, H., Tang, Q., Gao, X., Li, X., Lu, Y., Liu, B., Hu, C., & Li, X. (2018). Hepatic F-Box Protein FBXW7 Maintains Glucose Homeostasis Through Degradation of Fetuin-A. *Diabetes*, *67*(5), 818–830. https://doi.org/10.2337/db17-1348

Zhao, X., Hirota, T., Han, X., Cho, H., Chong, L.-W., Lamia, K., Liu, S., Atkins, A. R., Banayo, E., Liddle, C., Yu, R. T., Yates, J. R., Kay, S. A., Downes, M., & Evans, R. M. (2016). Circadian Amplitude Regulation via FBXW7-Targeted REV-ERBα Degradation. *Cell*, *165*(7), 1644–1657. https://doi.org/10.1016/j.cell.2016.05.012

Zheng, N., Wang, P., Jeffrey, P. D., & Pavletich, N. P. (2000). Structure of a c-Cbl-UbcH7 complex: RING domain function in ubiquitin-protein ligases. *Cell*, *102*(4), 533–539. https://doi.org/10.1016/s0092-8674(00)00057-x

Zhong, L., Zhang, Y., Li, M., Song, Y., Liu, D., Yang, X., Yang, D., Qu, H., Lai, L., Wang, Q., & Chen, Z. (2020). E3 ligase FBXW7 restricts M2-like tumor-associated macrophage polarization by targeting c-Myc. *Aging*, *12*(23), 24394–24423. https://doi.org/10.18632/aging.202293

Zhu, J., Li, Y., Chen, C., Ma, J., Sun, W., Tian, Z., Li, J., Xu, J., Liu, C. S., Zhang, D., Huang, C., & Huang, H. (2017). NF-κB p65 Overexpression Promotes Bladder Cancer Cell Migration via FBW7-Mediated Degradation of RhoGDIα Protein. *Neoplasia (New York, N.Y.)*, *19*(9), 672–683. https://doi.org/10.1016/j.neo.2017.06.002

CHAPTER 2

Suppression of the Ubiquitin Ligase Function of FBXW7 Accelerates Pancreatic Cancer Progression to Metastasis

Grissel Cervantes Jaramillo[1,2], Alex Jaeger[1,4], Emma Dawson[1,3], William Freed-Pastor[1], Javier Villa Ortiz[1,3], Richard Schiavoni[1,5], Emma Sanders[1], Sean-Luc Shanahan[1,3], Kim Mercer[1], Tyler E. Jacks[1,3]

Affiliations:

[1]Koch Institute for Integrative Cancer Research, Massachusetts Institute of Technology Cambridge, MA 02140

[2]Harvard-MIT Division of Health Sciences and Technology, Harvard Medical School, Massachusetts Institute of Technology, Cambridge, MA 02139

[3]Department of Biology, Massachusetts Institute of Technology Cambridge, MA 02140

[4]Currently, H. Lee Moffitt Cancer Center & Research Institute, Tampa, FL 33612

[5]Robert A. Swanson (1969) Biotechnology Center, Biopolymers & Proteomics Core, Koch Institute for Integrative Cancer Research, Massachusetts Institute of Technology Cambridge, MA 02140

Abstract

Pancreatic ductal adenocarcinoma (PDAC) is the most lethal common malignancy because it is usually diagnosed at an advanced or metastatic stage. Dysregulation of protein stability and degradation has been associated with uncontrolled proliferation and genomic instability, leading to cancer progression to metastasis. One of the major regulators of protein degradation is the tumor suppressor FBXW7, a substrate recognition domain of the SCF E3 ubiquitin ligase, frequently dysregulated in many cancers. The function and clinical significance of FBXW7 in PDAC have been studied in some detail, showing that FBXW7 acts as a tumor suppressor in PDAC cells. However, studies on the impact of FBXW7 expression and its substrates on pancreatic cancer progression to metastasis remain unclear. Here, we demonstrate that *Fbxw7* loss accelerates the progression and metastatic potential of pancreatic cancer in *Kras*$^{G12D/+}$; *Trp53*$^{-/-}$ PDAC models, in immunocompromised and immunocompetent hosts. We explore the impact of different Fbxw7 mutants in tumorigenesis, where the hotspot mutant R465 recapitulates the phenotype seen in complete loss-of-function of Fbxw7. Finally, we looked at global proteomic changes when Fbxw7 is lost to better understand mechanistically the role of Fbxw7 in PDAC progression to metastasis. This study presents a novel and mechanistically distinct facets of pancreatic cancer progression to metastasis.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the most lethal common malignancy and is currently the third leading cause of cancer deaths in the United States. The 5-year relative survival rate of all stages is 11.5%, mostly because 52% of patients are diagnosed at an unresectable and metastatic stage, for which the 5-year survival rate is only 3.1% (Howlader et al., 2021) and treatment options for these patients are very limited. The development of PDAC follows a stepwise progression driven by the gradual accumulation of genetic mutations. This progression is characterized by the transition of normal pancreatic ductal cells to a pre-invasive precursor lesion known as pancreatic intraepithelial neoplasia (PanIN) due to activating mutations of oncogenic *KRAS* (Hingorani et al., 2003; Kanda et al., 2012), which can ultimately develop into an invasive PDAC after inactivation of tumor suppressors such as *CDKN2A*, *TP53,* and *SMAD4* (Hustinx et al., 2005; Kanda et al., 2012; Morton et al., 2010; Wilentz et al., 2000). Typically, pancreatic cancer cells metastasize to the regional lymph nodes, then to the liver, and less commonly to the lungs, kidneys, and adrenal glands (Ryan et al., 2014). Despite the clinical importance of metastatic spread, our knowledge of the molecular processes that underlie PDAC's capacity for metastatic spread is still limited.

The ubiquitin-proteasome system regulates the abundance of various cellular proteins, vital for many cellular processes. As a result, its deregulation may result in uncontrolled proliferation and genomic instability, which can contribute to cancer development and metastasis. Ubiquitination is an enzymatic reaction that leads to the attachment of ubiquitin moieties to target proteins and to ubiquitin itself, creating

polyubiquitin chains. This process is orchestrated by the sequential activity of ubiquitin activation enzymes (E1), ubiquitin-conjugating enzymes (E2), and ubiquitin gases (E3) (Ciechanover, 2015; Hershko et al., 1979), eventually leading to proteasomal degradation of targeted proteins, as well as changes in protein activity, localization, or complex formation (Husnjak & Dikic, 2012; Popovic et al., 2014; Senft et al., 2018; Yau & Rape, 2016). Many E3s are frequently dysregulated in cancer through epigenetic and genetic mechanisms, as well as altered post-translational mechanisms that change in response to extrinsic and intrinsic cues (Qi & Ronai, 2015; Senft et al., 2018).

A major class of ubiquitin ligases is the Skp1-Cul1-F-box protein (SCF) complex, which consists of four components: the invariable subunits *SKP1*, *CUL1,* and *RBX1* and a variable F-box protein that affects target specificity by acting as a receptor for target proteins (Skaar et al., 2014; Winston et al., 1999). According to an analysis of the Catalogue of Somatic Mutations in Cancer (COSMIC) database, the F-box and WD-40-containing protein 7 (FBXW7) has the greatest mutation frequency among the several F-box proteins that have been found (Cenciarelli et al., 1999; Winston et al., 1999; Forbes et al., 2017). The most common mutations are found in mutational hotspots, R505, R465, and R479, codons located in the WD40 substrate binding domains, which may reduce its stability to form a stable SCF complex or may affect its interaction with specific substrates (Forbes et al., 2017; Yeh et al., 2018). There are three FBXW7 isoforms: FBXW7α, FBXW7β, and FBXW7γ, which localize to different sub-cellular compartments, further restricting interactions with specific partners and functions (Spruck et al., 2002). FBXW7α is ubiquitously expressed in the majority of proliferating cells and is localized in the nucleoplasm, FBXW7β is cytosolic and

FBXW7γ is nucleolar. Many of the known FBXW7 targets are proto-oncogenes such as cyclin E, MYC, MCL1, MTOR, JUN, NOTCH, and AURKA, which support the role of FBXW7 as a tumor suppressor (Fernandez et al., 2003; Foltz et al., 2002; Grim et al., 2008; Inuzuka et al., 2010; Koepp et al., 2001; Kwon et al., 2012; Mao et al., 2008; Wei et al., 2005; Yada et al., 2004).

The function and clinical significance of FBXW7 in pancreatic cancer have been studied in some detail, showing that FBXW7 acts as a tumor suppressor in pancreatic cancer (Calhoun et al., 2003; Gao et al., 2014; Ishii et al., 2017; Ji et al., 2015; Pérez-Mancera et al., 2012; Sancho et al., 2014; Ye et al., 2021; Zhang, Zhang, et al., 2016). FBXW7 ranks amongst the top 50 mutated genes in pancreatic cancer patients, with a mutation frequency of ~3.8% (TCGA Genome Data Analysis Center). In fact, pancreatic cancer patients with low FBXW7 expression levels have a poor probability of survival compared to patients with high FBXW7 expression levels (Ishii et al., 2017; Uhlén et al., 2005). Furthermore, studies have shown that *Fbxw7* mutations and loss cooperate with *Kras*^{G12D} to accelerate PDAC formation with a high frequency (24%), showing that *Fbxw7* is an important tumor suppressor in *Kras*-driven pancreatic cancer (Pérez-Mancera et al., 2012; Zhang, Zhang, et al., 2016). Furthermore, *in vitro* studies suggest that loss of *Fbxw7* function promotes cell proliferation, migration, and the epithelial-to-mesenchymal transition (EMT) needed for metastasis in PDAC (He et al., 2017; Ishii et al., 2017; Jin et al., 2017). However, studies on the impact of *Fbxw7* expression and its substrates on pancreatic cancer progression to metastasis are very limited.

Here, we demonstrate that *Fbxw7* loss accelerates metastatic progression of pancreatic cancer in *Kras*$^{G12D/+}$; *Trp53*$^{-/-}$ driven PDAC models, in immunocompromised and immunocompetent hosts. We show that loss of *Fbxw7* increases the metastatic potential of PDAC cells. Furthermore, we explore the impact of different Fbxw7 mutants in tumorigenesis, where only the hotspot mutant R465 recapitulates the phenotype seen in complete loss-of-function of Fbxw7. Finally, we looked at global proteomic changes when Fbxw7 is lost in an attempt to better understand mechanistically the role of Fbxw7 in PDAC progression to metastasis.

Results

Fbxw7 loss accelerates PDAC metastatic progression in immunocompromised and immunocompetent mouse models

We hypothesized that suppression of the ubiquitin ligase function of *Fbxw7* induces metastatic progression of pancreatic cancer. To test this hypothesis, we knocked out *Fbxw7* using CRISPR/Cas9 technology in the *Kras*$^{LSL\text{-}G12D}$; p53$^{flox/flox}$ model, which faithfully mimics human KRAS-driven PDAC, displaying similarities at the molecular and histopathological level (Hruban et al., 2000; Lee et al., 2019). We derived pancreatic organoids from normal pancreata of *Kras*$^{LSL\text{-}G12D/+}$; *Trp53*$^{flox/flox}$; *Rosa26*$^{LSL\text{-}Cas9/LSL\text{-}TdTomato}$ mice. Following *ex vivo* delivery of adenovirus containing Cre recombinase, pancreatic organoids express oncogenic Kras with loss of tumor suppressor p53, as well as expression of endonuclease Cas9 and the fluorescent protein TdTomato (KP Cas9/Tom). This system allows efficient CRISPR/Cas9 genomic editing, as well as *in vivo* tracking of cancer cells by fluorescence imaging. Organoids are three-dimensional cell clusters that self-organize into organ-like structures, that have been shown to recapitulate PDAC disease at the pathophysiological level (Boj et al., 2015). KP Cas9/Tom pancreatic organoids were transduced with lentiviral vectors expressing sgRNA against *Fbxw7* (sgFbxw7.1) and *Olfr102* as a control (sgOlfr102) (**Figure 1A**). Olfr102 was identified as a non-essential gene in PDAC in multiple shRNA screens. Editing at the *Fbxw7* locus was confirmed by sequencing and TIDE analysis, which quantifies INDEL rates and provides an estimate of gene editing efficiency, which in this case was 48.3% (**Figure 1A**).

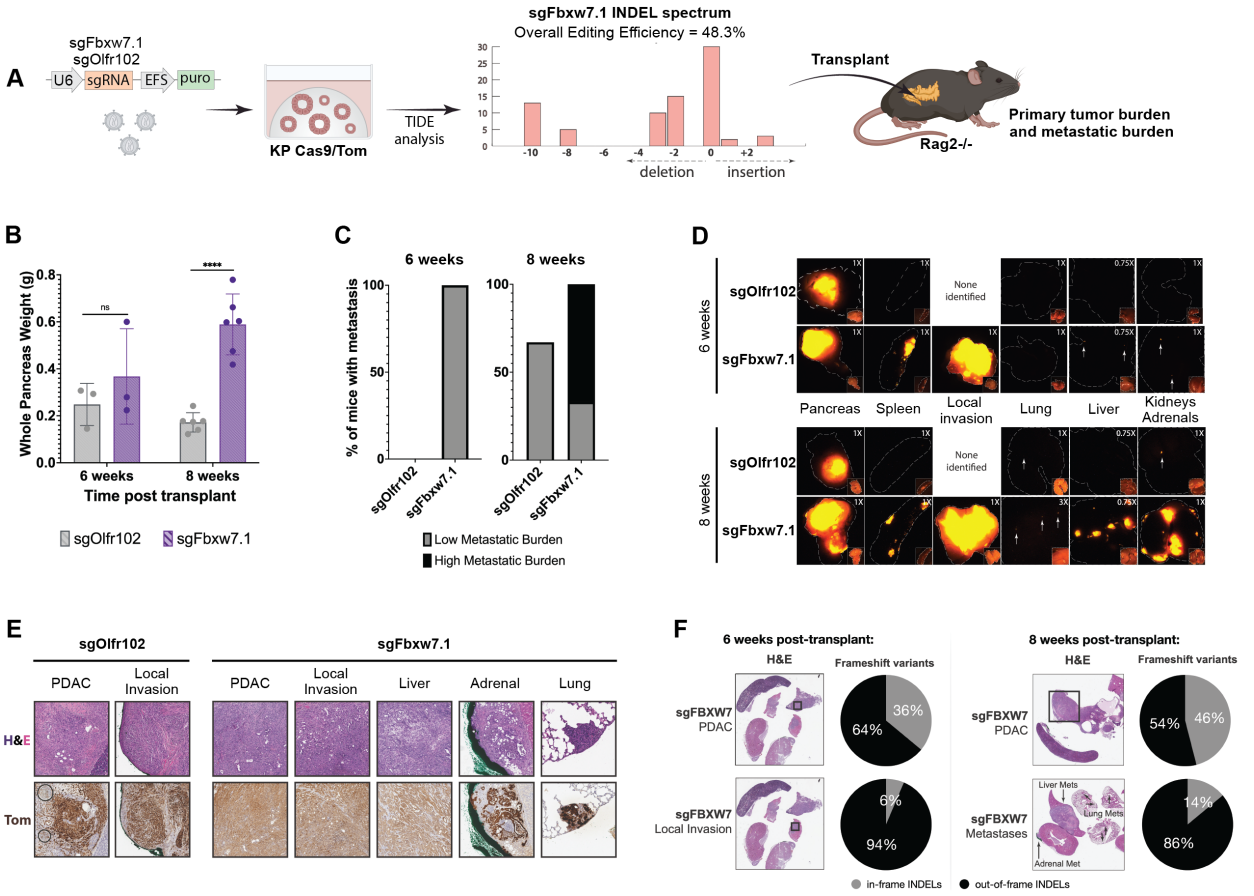


Figure 1: Loss-of-function of *Fbxw7* accelerates PDAC progression to metastasis in immunocompromised hosts.

(A) Experimental design. Briefly, KP Cas9/Tom organoids were transduced with lentivirus containing sgRNA against *Fbxw7* and control *Olfr102* for CRISPR/Cas9 editing. Editing efficiency was confirmed by sequencing and TIDE analysis, revealing a 48.3% overall editing efficiency at the *Fbxw7* locus. Organoids were orthotopically transplanted to Rag2$^{-/-}$ hosts and primary and tumor progression were assessed at 6 and 8 weeks post-transplant.

(B) Whole pancreas weight (grams) as a proxy for primary burden when Fbxw7 is lost compared to control animals at 6 and 8 weeks post transplant. **** $p<0.0001$.

(C) Percentage of animals with distal metastases in the liver, kidneys, adrenal glands, and lungs when *Fbxw7* is lost compared to control animals at 6 and 8 weeks post transplant.

(D) Representative TdTomato fluorescence images of the pancreas and distal organs show primary tumors and metastatic foci from *Fbxw7* knockout and control animals at 6 and 8 weeks post transplant. Arrows point to micrometastatic foci.

(E) Representative images of the histopathology of tumors from *Fbxw7* knockout and control animals. TdTomato staining marks tumors formed from the transplanted organoid lines.

(F) Distribution of *Fbxw7* in-frame and out-of-frame INDELs in laser captured primary and metastatic tumors.

To avoid tumor rejection due to the expression of antigenic proteins Cas9 and TdTomato, the organoid lines were orthotopically transplanted into immunocompromised Rag2$^{-/-}$ animals (**Figure 1A**), which revealed a significant increase in primary and metastatic tumor burden when *Fbxw7* function is lost (**Figure 1B-1D**). Histological analysis of these tumors revealed that TdTomato+ primary and metastatic tumors recapitulate the histopathology of the human disease from abnormal glands to more advanced and poorly differentiated tumors, as well as the characteristic desmoplastic stroma in PDAC (**Figure 1E**). Furthermore, to look at the distribution of INDELs at the *Fbxw7* locus in the targeted tumors, primary and metastatic tumors from sgFbxw7.1 mice were laser-capture microdissected, and DNA from these tumors were sequenced by next-generation sequencing. Interestingly, at both early (6 weeks post-transplant) and late (8 weeks post-transplant) stages of tumor progression, the primary tumors show an equivalent representation of in-frame and out-of-frame INDELs (**Figure 1F**). On the other hand, the invasions and metastatic tumors showed significant enrichment of out-of-frame INDELs that more likely lead to complete loss-of-function of *Fbxw7* (**Figure 1F**). Collectively, this data shows that loss of *Fbxw7* function accelerates PDAC progression to metastasis in immunocompromised mouse models of PDAC.

Accelerated PDAC metastatic progression is not linked to the loss of a single Fbxw7 isoform, but, rather, the complete loss of all Fbxw7 isoforms

In principle, the three *Fbxw7* isoforms (Fbxw7α, Fbxw7β and Fbxw7γ) are functionally identical since they share the three main functional domains: the dimerization domain (DD), the F-box domain which interacts with Skp1 from the SCF complex, and eight WD40 domains that interact with target proteins for polyubiquitination (Yeh et al., 2018, p. 7; Yumimoto & Nakayama, 2020) (**Figure 2A**). Distinct N-terminal signals in the isoform-specific first exon direct FBXW7α to the nucleoplasm, FBXW7 β to the cytoplasmic membranes and FBXW7γ to the nucleolus, which restricts the protein pool they sample for degradation (Bonetti et al., 2008; Durgan & Parker, 2010; Grim et al., 2008; Matsumoto et al., 2006; Welcker et al., 2004).

To test whether the observed accelerated PDAC progression to metastasis is linked to the loss of a single *Fbxw7* isoform, we used CRISPR/Cas9 technology to knockout each individual isoform (sgFbxw7α, sgFbxw7β and sgFbxw7γ), as well as to completely knockout all *Fbxw7* isoforms (sgFbxw7.1, which targets the C-terminal WD40 domains, and sgFbxw7.2, which targets upstream of all functional domains) (**Figure 2A**). Towards this goal, we derived genetically defined *Kras*$^{LSL\text{-}G12D/+}$; *Trp53*$^{flox/flox}$; *Rosa26*$^{LSL\text{-}Cas9\text{-}eGFP/LSL\text{-}Cas9\text{-}eGFP}$ (KP Cas9-GFP) pancreatic organoids, and *ex vivo* transformed them with adenovirus expressing Cre recombinase. Transformed organoids were transduced with lentiviral vectors expressing the sgRNA against *Fbxw7* variants and *Olfr102* control (sgOlfr102, sgFbxw7α, sgFbxw7β and sgFbxw7γ, sgFbxw7.1 and sgFbxw7.2) (**Figure 2A and S2A**). Organoid lines were cloned, and only clones with out-of-frame INDELs leading to homogenous knockouts were used (**Figure**

S2A and S2B). Orthotopic transplantation of these organoid lines into syngeneic, immunocompetent mice (C57BL/6 KP Cas9-GFP) revealed that only complete loss of Fbxw7 (sgFbxw7.1 and sgFbxw7.2) lead to a significant increase in primary and metastatic tumor burden (**Figure 2B–2D and S2C**). Together this data shows that there is isoform redundancy (isoforms compensate each other) and that only complete loss-of-function of all three *Fbxw7* isoforms accelerates PDAC tumor progression to metastasis.

To further explore the metastatic potential of PDAC cells when *Fbxw7* function is lost, sgOlfr102, sgFbxw7.1 and sgFbxw7.2 pancreatic organoid lines were adapted to 2D and followed up by *in vitro* and *in vivo* proliferation, migration, and invasion assays. While there was no significant difference observed in their *in vitro* proliferation and migration capacity (**Figure 2E and S2D–S2F)**, *in vivo* tail vein injection of these lines revealed a significant increase in lung colonization when *Fbxw7* is lost (**Figure 2E and 2F**), demonstrating an increased metastatic potential of PDAC when *Fbxw7* is lost.

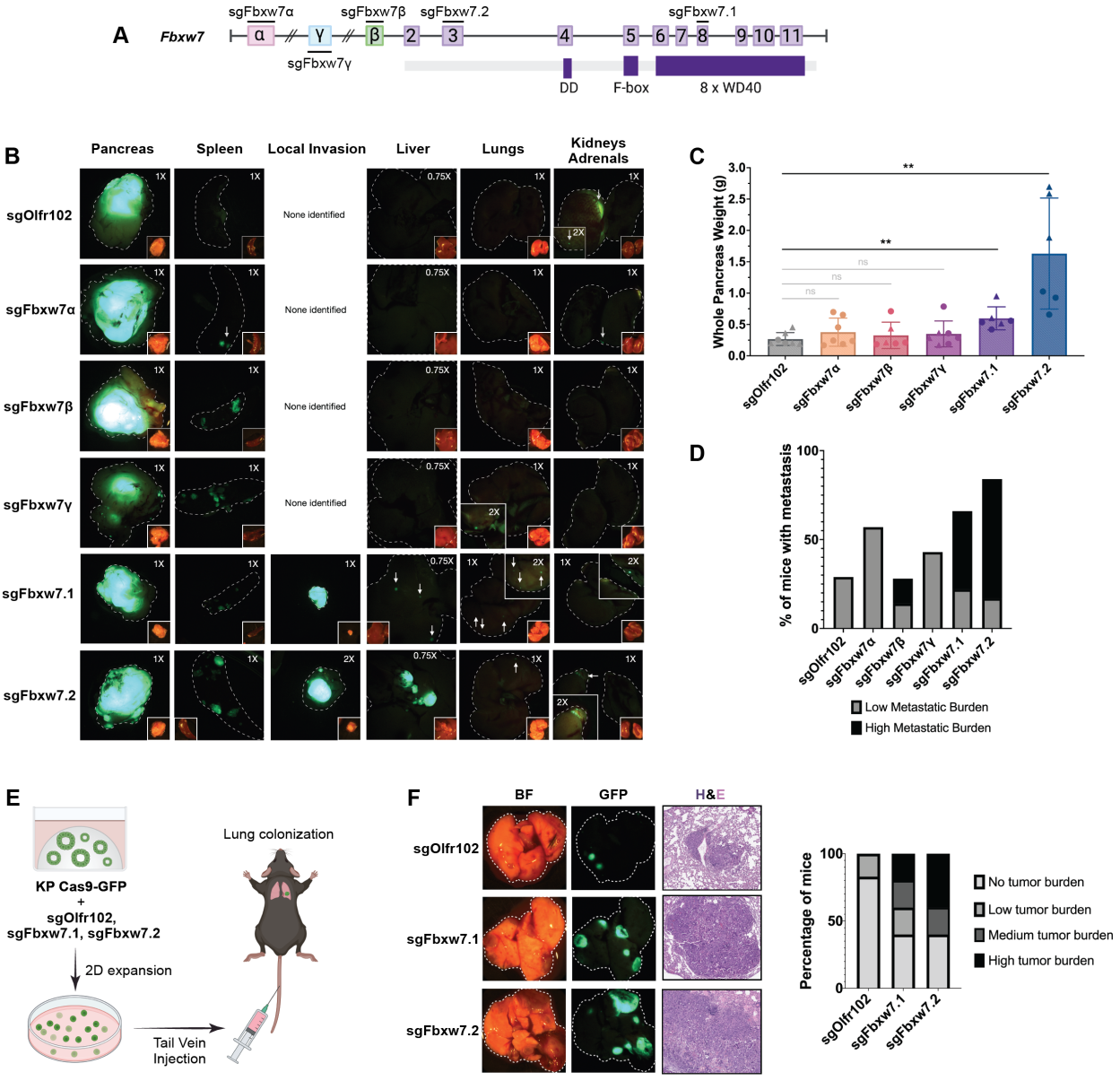


Figure 2: Interrogation of the the different Fbxw7 isoforms in PDAC progression to metastasis.

(A) Representative diagram of the Fbxw7 genomic locus and functional domains (DD, dimerization domain; F-box domain; WD40 repeats, substrate binding domains). sgRNAs targeting loci used to target each individual Fbxw7 isoform (sgFbxw7α, sgFbxw7β and sgFbxw7γ), and all isoforms simultaneously (sgFbxw7.1 and sgFbxw7.2).

(B) Representative GFP fluorescence images of the pancreas and distal organs show primary tumors and metastatic foci from Fbxw7α, Fbxw7β, Fbxw7γ, Fbxw7.1 and Fbxw7.2 knockout animals at 9 weeks post transplant. Arrows point to micrometastatic foci.

(C) Whole pancreas weight (grams) as a proxy for primary burden from Fbxw7α, Fbxw7β, Fbxw7γ, Fbxw7.1 and Fbxw7.2 knockout animals at 9 weeks post transplant.. ** $p<0.01$.
(D) Percentage of animals with distal metastases in the liver, kidneys, adrenal glands, and lungs from Fbxw7α, Fbxw7β, Fbxw7γ, Fbxw7.1 and Fbxw7.2 knockout animals at 9 weeks post transplant.
(E) Experimental design of tail vein injections. Briefly, Fbxw7.1 and Fbxw7.2 knockout organoids were transiently adapted to 2D cultures, expanded and 100,000 cells were injected via tail vein. Lung colonization was analyzed by fluorescence imaging and histology.
(F) Representative GFP fluorescence images and histology of lung tumors after tail vein injection of Fbxw7.1 and Fbxw7.2 knockout lines. Percentage of animals with low, medium or high lung tumor burden.

Fbxw7 loss leads to stabilization of c-Myc in PDAC tumors

So far, we have shown that *Fbxw7* loss increases the metastatic potential of PDAC, leading to accelerated progression in both immunocompromised and immunocompetent mouse models of PDAC. In order to mechanistically interrogate the role of *Fbxw7* in PDAC progression, we started by establishing a clean knockout system by introducing a Cre recombinase conditional *Fbxw7* allele (Fbxw7$^{flox/flox}$; F) to the Kras$^{LSL\text{-}G12D/+}$; Trp53$^{flox/flox}$; R26$^{LSL\text{-}TdTomato}$ (KPT) mouse model (**Figure 3B)**. This Fbxw7$^{flox/flox}$ allele contains LoxP sites flanking exons 5 and 6, which encode the F-box domain and WD40 repeats, leading to complete deletion of *Fbxw7* after Cre-mediated recombination (**Figure 3B**) (Thompson et al., 2008). We derived genetically defined pancreatic organoids from normal pancreata of mice C57BL/6 KPT and KPFT mice (**Figure 3A**). Following *ex vivo* transformation by delivery of adenovirus expressing Cre recombinase, these organoids express oncogenic *Kras* with loss of tumor suppressor *p53* and *Fbxw7* (in the case of KPFT organoids), as well as expression of the fluorescent protein TdTomato (**Figure 3A, 3B and S2A**). Similar to what we have shown

before, these organoids show no difference in their *in vitro* proliferative capacity (**Figure 3C, S2B–S2D**), but upon orthotopic transplantation to syngeneic mice, there is a significant increase in PDAC primary and metastatic tumor burden when *Fbxw7* is lost (**Figure S2E–S2G**). Furthermore, KPFT mice have a significantly lower probability of survival with a median survival of 8.3 weeks post-transplant, while KPT mice have a median survival of 10.5 weeks post-transplant (**Figure 3D**). Histologic analysis of TdTomato+ tumors show similar histopathological features of the human disease, from abnormal glands to more advanced and poorly differentiated tumors in the case of KPFT mice (**Figure 3E**). In addition, immunohistochemistry staining of known Fbxw7 targets revealed a significant accumulation of c-Myc in KPFT mice compared to KPT controls (**Figure 3E and 3F**).

To further explore the role of *Fbxw7* in metastatic progression of PDAC, we measured circulating tumor cell (CTC) shedding longitudinally, over 2 weeks during tumor progression in the same unanesthetized mouse using an optofluidic device (Hamza et al., 2019). Interestingly, KPFT mice showed lower numbers of CTCs normalized to primary tumor burden, compared to KPT mice, which showed variable higher numbers (**Figure 4A**). Moreover, tail vein injections of 2D adapted KPFT and KPT cells showed an increase in lung colonization (**Figure 4B and 4C**). Collectively, this suggests that KPFT CTCs have a higher potential of extravasation and/or foreign organ colonization, increasing their metastatic potential. However, further studies are required to dissect the role of *Fbxw7* in the metastatic cascade or PDAC.

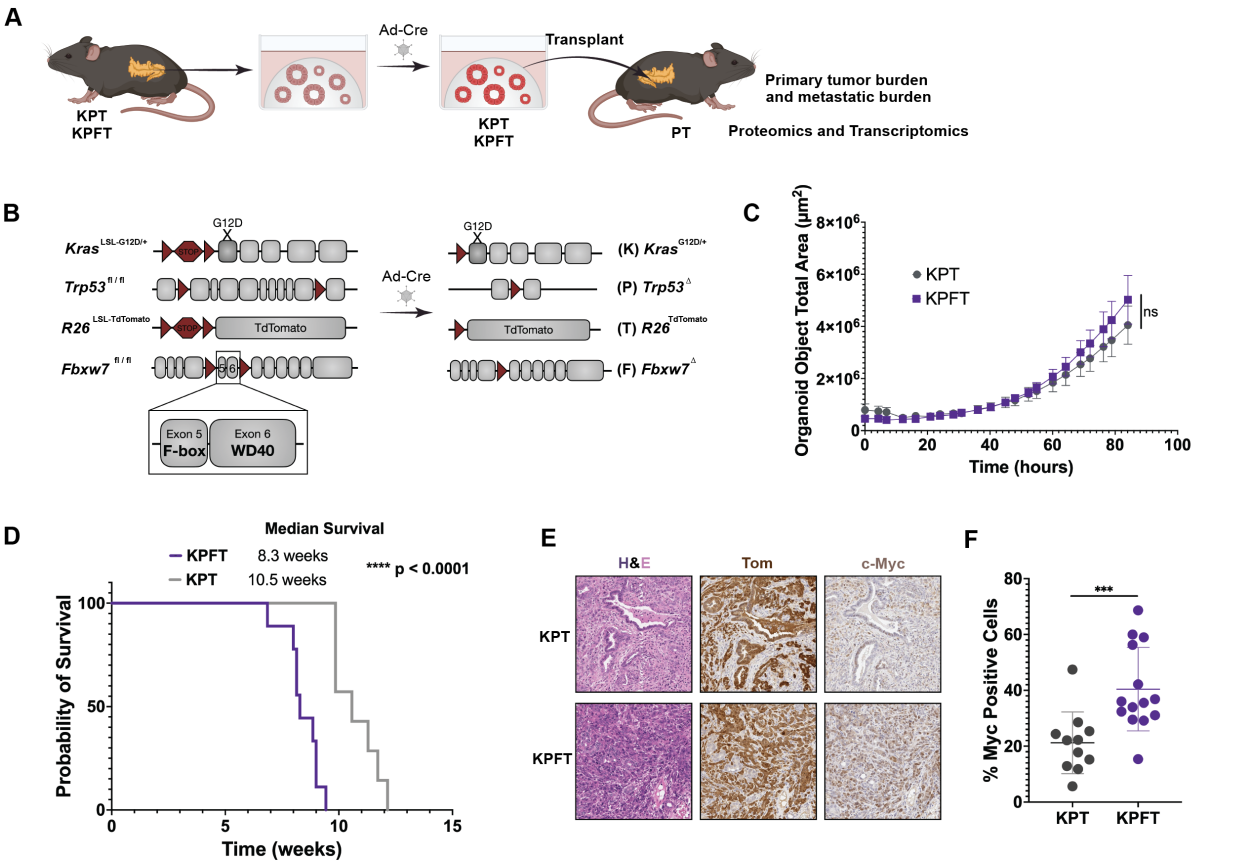


Figure 3: Cre-mediated deletion of Fbxw7 recapitulates accelerated PDAC tumor progression and leads to increased metastatic potential.

(A) Experimental design for the establishment of pancreatic organoids from KPT (Kras$^{LSL\text{-}G12D/+}$; Trp53$^{flox/flox}$; R26$^{LSL\text{-}TdTomato}$) and KPFT (Kras$^{LSL\text{-}G12D/+}$; Trp53$^{flox/flox}$; R26$^{LSL\text{-}TdTomato}$; Fbxw7$^{flox/flox}$) animals. This is followed by *ex-vivo* transformation with adenovirus expressing Cre recombinase and Nutlin treatment for the selection of successful transformation. Organoids were orthotopically transplanted to immunocompetent (PT) hosts.

(B) Representative diagrams of the Cre-conditional alleles used in this study: Kras$^{LSL\text{-}G12D/+}$; Trp53$^{flox/flox}$; R26$^{LSL\text{-}TdTomato}$ and Fbxw7$^{flox/flox}$

(C) Total organoid area over time of KPT and KPFT organoids as a measure of proliferation.

(D) Kaplan-Meier curve showing the probability of survival of animals after orthotropic transplant of KPT and KPFT organoid, accompanied by the median survival time for each experimental group. **** p<0.0001.

(E) Histopathology of KPT and KPFT tumors (H&E). Immunohistochemistry staining of TdTomato, which marks tumors from the transplanted organoid lines, and c-Myc.

(F) Percentage of cells positive for c-Myc expression from IHC staining of KPT and KPFT tumors.

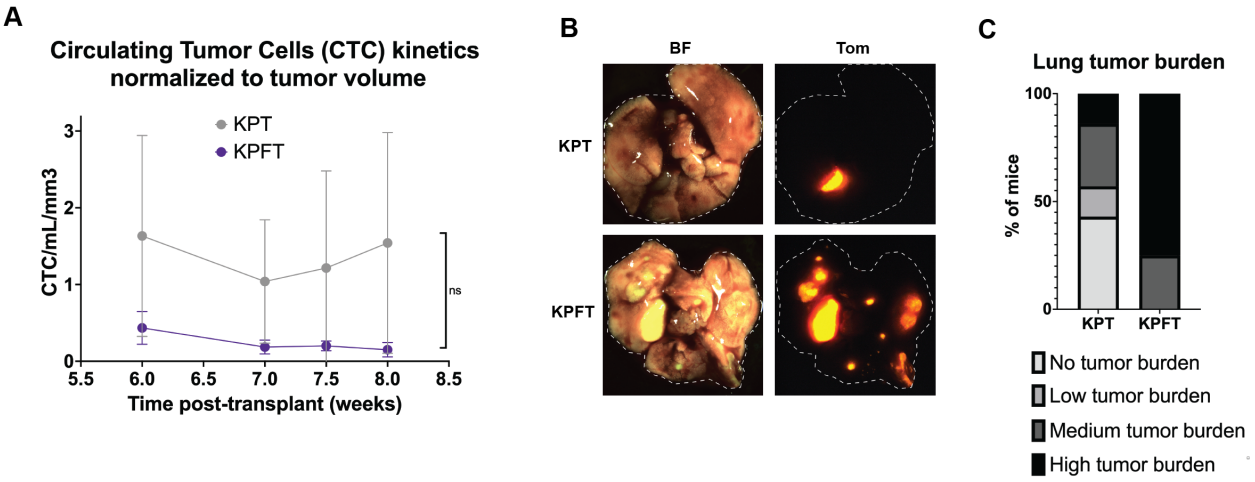


Figure 4: Fbxw7 loss increases the metastatic potential of PDAC cells.
(A) Number of circulating tumor cells (CTCs) per mL of blood sampled, normalized to the tumor volume of KPT and KPFT animals (n=3 per group)
(B) Representative TdTomato fluorescence images of lungs after tail vein injection of KPT and KPFT cells.
(C) Percentage of mice of low, medium and high lung tumor burden after tail vein injection of KPT and KPFT cells.

The majority of Fbxw7 targets are known proto-oncogenes, leading to the degradation of cell cycle activators and oncogenic proteins such as Cyclin E, c-Myc, Aurora-A, Notch, c-Jun, and HIF-1a. Therefore, we looked at global proteomic and transcriptomic changes in KPFT and KPT tumors, in order to deeply understand the role of Fbxw7 in the metastatic progression of PDAC. Towards this goal, tumor cells were isolated from KPFT and KPT mice by fluorescence-activated cell sorting (FACS) of TdTomato+ tumor cells from late-stage tumors (8 weeks post-transplant), followed by mass spectrometry and RNA-sequencing. Differential protein expression analysis followed by gene set enrichment analysis revealed pathways that are downregulated or enriched in KPFT tumors compared to KPT control tumors (**Figure 5A and 5B**). These studies revealed a significant enrichment of c-Myc targets, hypoxia response pathways

and the unfolded protein response in KPFT tumors, as expected based on known *Fbxw7* targets. Interestingly, KPFT tumors show consistent downregulation of inflammatory processes such as interferon alpha and gamma response, as well as downregulation of antigen processing and presentation. This raises interesting biology to be further explored since recent studies have shown that Fbxw7 loss impairs dsRNA sensing pathways, leading to impaired production of type I interferons and an altered tumor microenvironment (Gstalder et al., 2020; Song et al., 2017). Furthermore, gene ontology analysis of the most dysregulated proteins revealed downregulation of glutathione metabolic processes and an enrichment of hydrogen peroxide catabolic processes, iron metabolism, and oxygen transport.

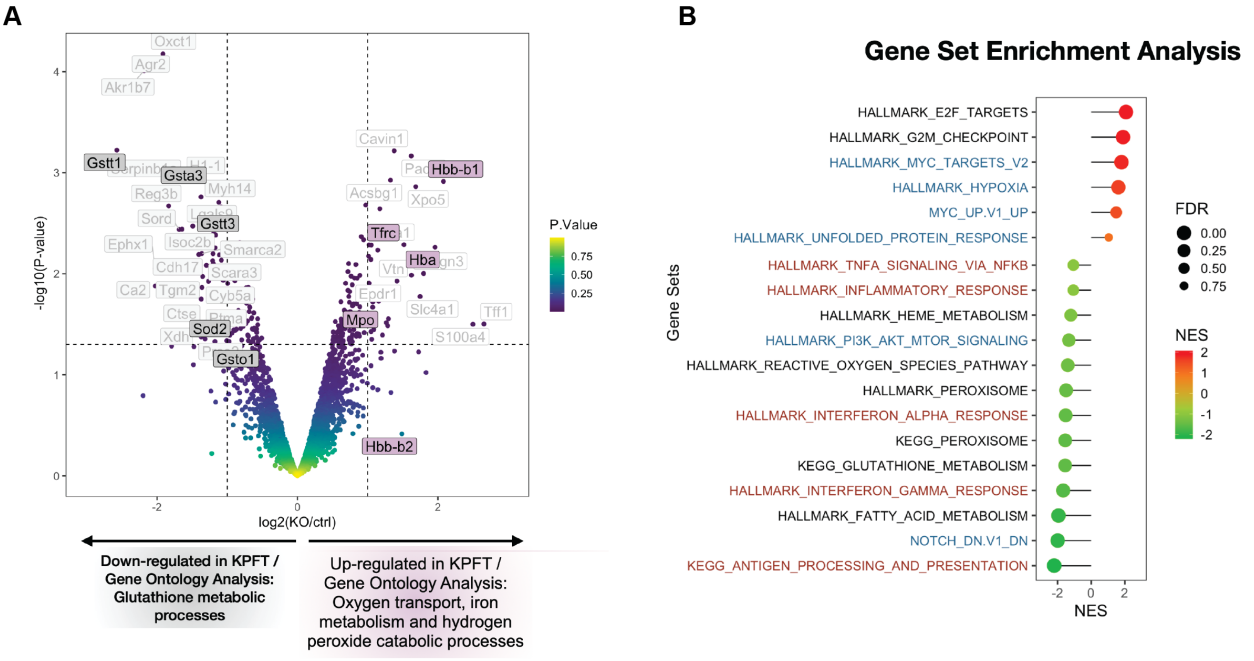


Figure 5: Global proteomic changes after loss of Fbxw7 in PDAC tumors.

(A) Differential protein expression analysis (DEqMS) between KPFT and KPT PDAC tumors is represented in a volcano plot. Bottom panel shows gene ontology analysis of the most differentially expressed proteins with a log2(KO/ctrl)>1 and -log10(p-value)>1.3.

(B) Gene set enrichment analysis (GSEA) of DEqMS data in KPFT tumors compared to KPT tumors. NES, Normalized Enrichment Score; FDR, false discovery rate.

Conditional reexpression of Fbxw7 regresses phenotype

Aiming to further dissect the role of *Fbxw7* in pancreatic tumorigenesis and metastasis, we developed a system to conditionally re-express wild type Fbxw7, as well as hotspot mutants R505 and R465 (Forbes et al., 2017; Yeh et al., 2018), in the *Fbxw7* knockout organoids, and track how this affects PDAC metastatic progression. Towards this goal, we designed a doxycycline-inducible TetON system to regulate Fbxw7 re-expression in tumors. These constructs have an EF1α promoter driving constitutive expression of the transcriptional activator (rtTA), and a doxycycline-responsive promoter TRE3G driving expression of FLAG-tagged inserts of interest: Fbxw7^{WT}-FLAG, Fbxw7^{R465C}-FLAG, Fbxw7^{R505C}-FLAG, Fbxw7$^{\Delta WD40}$-FLAG and no insert control (NIC) (**Figure 6A**) (Fbxw7$^{\Delta WD40}$ has functional DD and F-box domains but lacks all WD40 repeats). Transduction of KPFT and KPT organoids with lentivirus containing these constructs, leads to stable organoid lines with the ability to temporally regulate the expression of the inserts by adding doxycycline, both *in vitro* and *in vivo* (**Figure 6A**). Proof of concept of the system *in vitro* revealed increasing expression of FLAG-tagged Fbxw7^{WT} when doxycycline is given, in a dose-dependent manner (**Figure 6B**). Interestingly, c-Myc levels immediately decrease by ~75% when doxycycline is added (**Figure 6B**), suggesting that low levels of Fbxw7 expression is sufficient to target c-Myc for degradation, and that it is not dose-dependent.

The established organoid lines (KPT NIC, KPFT NIC, KPFT + Fbxw7^{WT}, KPFT + Fbxw7^{R465C}, KPFT + Fbxw7^{R505C,} and KPFT + Fbxw7$^{\Delta WD40}$) were orthotopically transplanted into immunocompromised Rag2$^{-/-}$ mice, and doxycycline diet was given to mice upon transplantation. Longitudinal ultrasound tracking of PDAC tumors

revealed that KPFT NIC tumor volume ($V_{avg.}$= 247.7 mm^3 at 6 weeks post-transplant) is significantly larger compared to KPT NIC tumors ($V_{avg.}$= 0 mm^3 at 6 weeks post-transplant), as expected from previous data, which is then rescued by the re-expression of $Fbxw7^{WT}$ (KPFT + $Fbxw7^{WT}$; $V_{avg.}$ = 0 mm^3 at 6 weeks post-transplant) (**Figure 6C**). Interestingly, the hotspot mutant $Fbxw7^{R505C}$ does not rescue the phenotype ($V_{avg.}$ = 303.4 mm^3 at 6 weeks post-transplant), meaning that these tumors recapitulate the accelerated progression observed with the complete loss-of-function of Fbxw7 (KPFT NIC) (**Figure 6D**). On the other hand, expression of mutants $Fbxw7^{R505C}$ ($V_{avg.}$ = 0 mm^3 at 6 weeks post-transplant) and $Fbxw7^{\Delta WD40}$ ($V_{avg.}$ = 28.03 mm^3 at 6 weeks post-transplant) behaved similar to expression of $Fbxw7^{WT}$ (**Figure 6D**). The fact that $Fbxw7^{\Delta WD40}$ expression does not recapitulate the phenotype seen in KPFT NIC raises interesting biology of *Fbxw7*, suggesting that *Fbxw7* plays a role in PDAC progression outside of its main function as an E3 ubiquitin ligase.

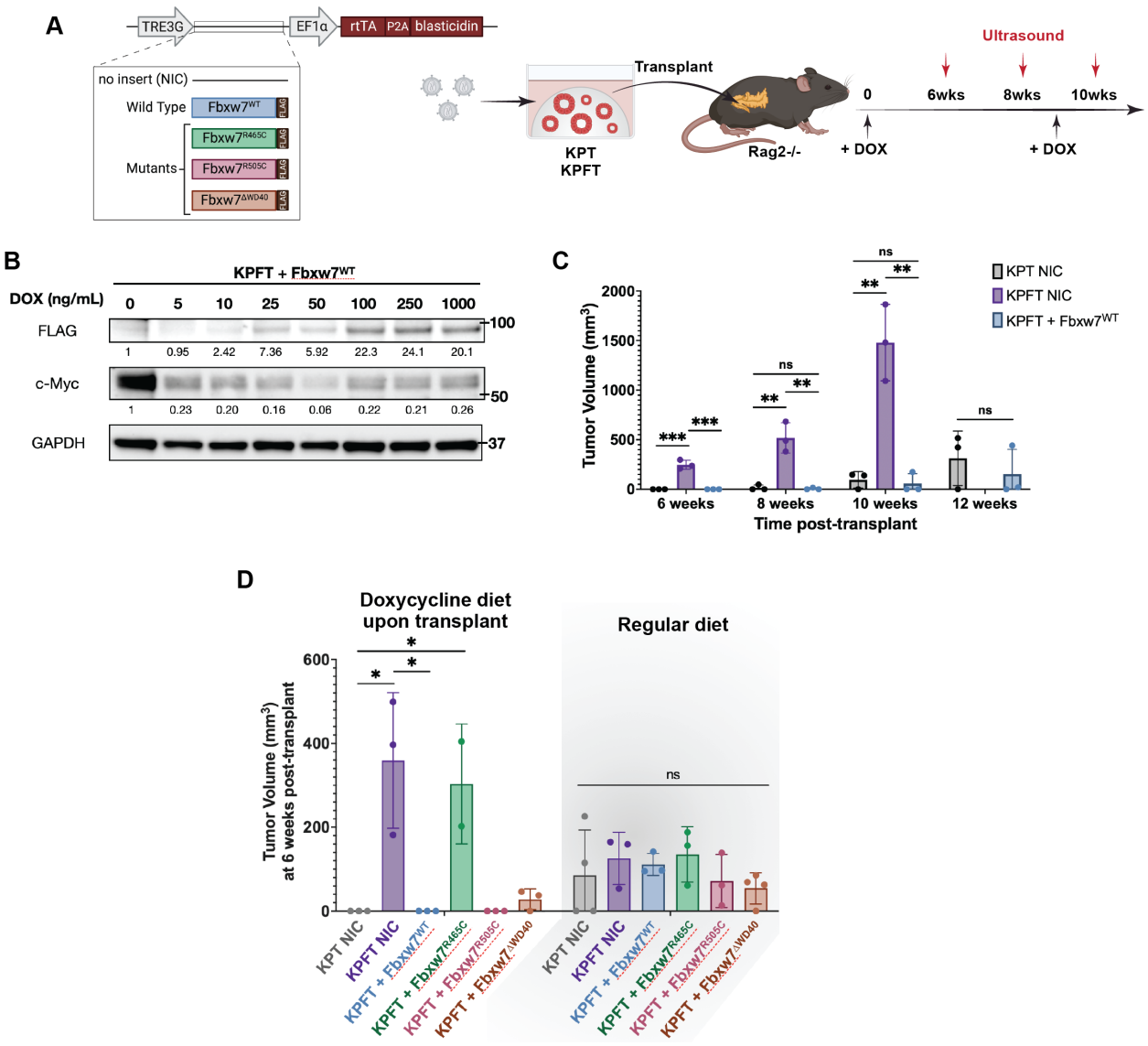


Figure 6: Conditional re-expression of wild-type Fbxw7 regresses phenotype.

(A) Experimental design. KPT and KPFT organoids were transduced with lentivirus expressing a doxycycline-inducible system for the temporal regulation of Fbxw7^{WT}, Fbxw7^{R465C}, Fbxw7^{R505C} and Fbxw7$^{\Delta WD40}$ expression. Organoids were orthotopically transplanted to Rag2-/- mice, and mice were given doxycycline food upon transplant. Tumor burden was tracked via ultrasound. NIC, no insert control.

(B) Representative western blot of KPFT+ Fbxw7^{WT}-FLAG organoid line exposed to increasing concentration of doxycycline (0-1000 µg/mL). Normalized band intensity quantification is shown below each panel.

(C) Longitudinal tumor volume was measured by ultrasound at 6, 8, 10 and 12 weeks post transplant of KPT NIC, KPFT NIC and KPFT+ Fbxw7^{WT} organoids. Doxycycline was given to all mice upon transplant. ** $p<0.01$

(D) Tumor volume measured by ultrasound at 6 weeks post transplant of KPT NIC, KPFT NIC, KPFT+Fbxw7^{WT}, KPFT+Fbxw7^{R465C}, KPFT+Fbxw7^{R505C}, and KPFT+Fbxw7$^{\Delta WD40}$ organoids. Left panel shows tumor volume with doxycycline upon transplant and right panel shows tumor volume without doxycycline. * $p<0.05$.

Discussion

Studies have explored the role and clinical significance of *Fbxw7* in pancreatic cancer to some extent, showing that Fbxw7 is an important tumor suppressor in Kras-driven PDAC (Calhoun et al., 2003; Gao et al., 2014; He et al., 2017; Ishii et al., 2017, 2017; Ji et al., 2015; Jin et al., 2017; Pérez-Mancera et al., 2012; Sancho et al., 2014; Ye et al., 2021; Zhang, Zhang, et al., 2016). However, the function of Fbxw7 dysregulation and its substrates in metastatic progression of PDAC remains unclear. Here, we interrogate this mechanically distinct pathway in PDAC progression to metastasis.

In this study we demonstrate that *Fbxw7* loss accelerates PDAC progression to metastasis in the KP mouse model (*Kras*$^{LSL\text{-}G12D/+}$; *Trp53*$^{fl/fl}$) in both immunocompromised and immunocompetent backgrounds. We show that while *Fbxw7* loss does not significantly impact proliferation and migration of PDAC organoids *in vitro*, it does lead to increased tumor colonization in distal organs *in vivo*, by tail vein injections. Furthermore, we show that only complete loss-of-function of all *Fbxw7* isoforms leads to accelerated metastatic progression of PDAC, while isoforms can compensate for the loss of any one of them.

We show that this phenotype is rescued by re-expression of wild type *Fbxw7*, while the expression of the hotspot mutant Fbxw7^{R465C} recapitulates the phenotype seen in complete loss-of-function of *Fbxw7*. Interestingly, Fbxw7$^{\Delta WD40}$ expression, which lacks the substrate recognition domains (ΔWD40), but still has functional DD and F-box domain, does not recapitulate the phenotype seen with complete loss-of-function of *Fbxw7*. This suggests that *Fbxw7* plays other functions in PDAC

progression in addition to supplying substrates for polyubiquitination and degradation. In fact, non-canonical functions of *Fbxw7* including complex formation, target protein stabilization, DNA damage repair and cell proliferation, have been previously described (Lan et al., 2019; Li et al., 2018; Song et al., 2017, p. 2; Zhang, Karnak, et al., 2016). Future studies could further dissect the different functional roles of *Fbxw7* in PDAC progression.

Moreover, we performed proteomic studies on *Fbxw7* knockout tumors, which revealed an enrichment of c-Myc targets, hypoxia response pathways, the unfolded protein response, and hydrogen peroxide catabolic processes. The data also shows consistent downregulation of inflammatory processes such as interferon alpha and gamma response and antigen processing and presentation, as well as glutathione metabolic processes. These dysregulated pathways raise interesting biology of *Fbxw7* in pancreatic cancer to be further explored. Collectively, data suggests that the dysregulation of multiple of these pathways potentially synergies to accelerate PDAC progression to metastasis. However, more studies are needed to dissect the mechanism of action.

In summary, this study presents novel and mechanistically distinct facets of pancreatic cancer progression to metastasis, which we envision will lead to more mechanistic studies with the potential to uncover novel therapeutic strategies for pancreatic cancer patients.

Materials and Methods

Mice:

All animal studies described in this study were approved by the MIT Institutional Animal Care and Use Committee. Mouse strains in this study were previously published: *Kras*$^{LSL\text{-}G12D}$ (Jackson et al., 2001), *Trp53*^{flox} (Marino et al., 2000), *FBXW7*^{flox} (Thompson et al., 2008), *Rosa26*$^{LSL\text{-}tdTomato}$ (Madisen et al., 2010) and Rag2$^{-/-}$ (Hao & Rajewsky, 2001). Mice were maintained in a C57BL/6 genetic background and bred in-house. FBXW7$^{flox/flox}$ and Rag2$^{-/-}$ were purchased from The Jackson Laboratory (JAX) and bred in-house. Tumors were initiated by pancreas orthotopic transplant surgery (see details below). When needed for experimental design, animals were fed with 625 ppm doxycycline rodent diet (Harlan-Tekla, catalog no. TD.01306). Animal health was monitored daily by the investigators and/or veterinary staff at the Department of Comparative Medicine at MIT. Mice were euthanized by CO_2 inhalation or cervical dislocation at defined time points (6, 8, or 10 weeks post-transplant) or upon reaching a body condition score under 2 for long-term studies.

Organoid generation and propagation:

Pancreatic organoid generation and propagation as previously described (Boj et al., 2015). Briefly, to generate pancreatic organoids from genetically defined mice, the pancreas was manually minced with razor blades and dissociated in digestion buffer (1X PBS, 125 U/mL collagenase IV, Worthington) for 20-30mins at 37^{o}C. Cells were filtered through 70μm cell strainers, washed with PBS, and centrifuged with slow deceleration. Cell pellets were embedded in 85-100% Matrigel (Corning) and solidified

at 37°C for 30mins. Subsequently, cells were cultured in complete medium AdDMEM/F12 medium supplemented with HEPES (1x, Invitrogen), GlutaMAX (1x, Invitrogen), penicillin/streptomycin (1x, VWR), B27 (1x Invitrogen), R-Spondin1-Conditioned Medium (Cultrex HA, R&D Systems, as per manufacturer's instructions) (10% v/v), A83-01 (0.5μM, Tocris), murine Epidermal Growth Factor (mEGF, 0.05μg/mL, PeproTech), Fibroblast Growth Factor 10 (FGF-10, 0.1μg/mL, PeproTech), Gastrin I (0.01μM, Tocris), recombinant murine Noggin (0.1μg/mL, PeproTech), N-acetyl-L-cysteine (1.25mM, Sigma-Aldrich), Nicotinamide (10mM, Sigma-Aldrich) and Y-27632 (10.5μM, Cayman Chemical Company, added right before use when organoids are thawed, or when organoids are dissociated to single cells) (minor modifications from previously described formulation (Boj et al., 2015)). Organoids were passaged with TrypLE Express (Life Technologies) for at least 4 passages to purify the ductal cells before proceeding with experiments.

For Cre recombinase-meditated recombination, organoids were spinfected with adenovirus Ad5-CMV-Cre at a MOI>100. Briefly, dissociated organoids and virus at desired MOI were centrifuged at 1700 rpm for 1 hour at room temperature, followed up by a 6 hour incubation at 37°C. Cells were then embedded in 100% Matrigel and cultured as described above. Recombination was confirmed by genotyping at the *Kras*, *Trp53* and *Fbxw7* loci.

For single organoid cloning, whole organoids were dissociated from Matrigel using dispase (1X, Corning) and plated individual organoids. Briefly, matrigel domes were incubated with dispase for 20-30 mins at 37°C, and then diluted with PBS. Using a pipette, individual organoids were picked under a microscope and transferred to a

well of a 96 well V-bottom plate. Organoid clones were resuspended in 30μL of 100% Matrigel and plated in 96 well flat bottom plates. Organoid clones were passaged as previously described.

Pancreas Orthotopic Transplants:

Orthotopic transplants of pancreatic organoids were performed with minor modifications from previously described protocols (Boj et al., 2015). Briefly, animals were anesthetized with Isofluorane, the left abdominal side was depilated with Nair and the surgical region was disinfected with Chloraprep swabstick (BD). A small incision (~1.5cm) was made in the left subcostal area and the spleen and pancreas were exteriorized with ring forceps. The organoid suspension (containing $1x10^5$ organoid cells in 100μL of 50% PBS + 50% Matrigel) was injected using a 30-gauge needle into the pancreatic parenchyma parallel to the main pancreatic artery. Successful injection was verified by the appearance of a fluid bubble without signs of intraperitoneal leakage. The pancreas and spleen were gently internalized, and the peritoneal and skin layers were sutured independently using 4/0 PGA suture and 4/0 silk suture respectively (AD Surgical). All mice received pre-operative analgesia Buprenorphine Sustained-Release (Bup-SR, 0.5mg/kg) and were followed up post-operatively for any signs of distress. Organoids/Matrigel mixtures were kept on ice throughout the whole procedure to avoid solidification. Male pancreatic organoids were only transplanted into male recipients.

Mice ultrasound:

Tumor volume quantification by high resolution ultrasound as previously described (Sastra & Olive, 2013). Briefly, animals were anesthetized by Isofluorane and the lateral and ventral abdominal areas were depilated using Nair. To improve visualization of the pancreas, sterile 0.9% saline (1mL) was administered by intraperitoneal injection. Imaging was done using the Vevo3100/LAZRX ultrasound and photoacoustic imaging system (Fujifilm-Visualsonics). Animals were placed on the imaging platform in the supine position and a layer of ultrasound gel was applied to whole depilated abdominal area. The ultrasound transducers (VisualSonics 550S and/or 250S) were placed on the abdomen orthogonal to the plane of the platform.

The area of the pancreas was defined by identifying landmark organs such as the kidney, spleen, and liver. The transducer was placed at the scanning midpoint of the normal pancreas or pancreatic tumor, and a 3D image of 10-20 mm was obtained at a Z- slice thickness of 0.04 mm, depending on tumor size. 3D images were loaded to the Vevo Lab Software and the volumetric analysis function was used to define the tumor border at various Z-slices throughout the tumor and calculate the final tumor volume. (As shown in Freed-Pastor et al., 2021).

Molecular cloning:

Lentiviral vectors (LV-U6-sgRNA-EFS-puromycin, LV-TRE3G-FBXW7-EF1α-rtTA-P2A-blasticidin and LV-TRE3G-EF1α-rtTA-P2A-blasticidin) were generated using gBlocks (IDT) and Gibson assembly. To insert sgRNAs, the vector was digested with FastDigest Esp3I (Thermo Fisher) and ligated using BsmBI-compatible annealed

oligonucleotides. sgRNAs were designed and assessed for off-target effects using Benchling (www.benchling.com).

Name	Protospacer Sequence
sgFbx7α	GAGCAAAAGACGACGAACTGG
sgFbxw7β	GTATGTCACAGATTCTAACG
sgFbxw7γ	GGAAAAGCTCCTGTGAACCA
sgFbxw7.1	ACGTTAGTGGGACATACAGG
sgFbxw7.2	GTGTTGCTGAACATGGTACA

Lentiviral production:

Lentiviruses were produced by co-transfection of HEK293 cells with lentiviral plasmids and packaging vectors PsPax2 (gift from Didier Trono - Addgene plasmid # 12260 ; http://n2t.net/addgene:12260 ; RRID:Addgene_12260) and Pmd2.G (gift from Didier Trono - Addgene plasmid # 12259 ; http://n2t.net/addgene:12259 ; RRID:Addgene_12259) at a 4:3:1 ratio. All plasmids were prepared using endotoxin-free midiprep kits (QIAGEN). Briefly, 7.5 x10^6 HEK293 cells were seeded in 15cm plates (Corning), 24 hours later cells were transfected using Mirus transfection reagent, and the media was replaced 24 hours after transfection. Viral supernatant were harvested at 48 and 72 hours post-transfection, filtered through a 0.45μm low-protein binding PVDF filter (EMD Millipore), and concentrated by ultracentrifugation at 25,000 rpm for 2 hours at 4°C. Concentrated virus was resuspended in Opti-MEM (Gibco) overnight at 4°C and aliquots were stored at -80°C.

Histology and Immunohistochemistry:

Tissues were fixed overnight in zinc formalin within 1 hour of necropsy, transferred to 70% ethanol, and embedded in paraffin. Sections were cut at 4-μm thickness and stained for hematoxylin and eosin (H&E) for histologic examination. For immunohistochemical analysis (IHC), unstained slides were dewaxed and antigen retrieval was done with citrate buffer (pH=6). Slides were blocked using the Endogenous Peroxidase Block (Dako) according to manufacturer's instructions, and endogenous species proteins were blocked using the appropriate species serum (Vector Labs) depending on the secondary antibody for 1 hour at room temperature. Slides were incubated with primary antibody overnight at 4°C. Primaries antibodies used were: anti-TdTomato (anti-RFP, Rockland, catalog no. 600-401-379, 1:400), anti-GFP (Novus Biological, catalog no. NB600-308SS, 1:1000), anti-Myc (Abcam, catalog no. ab32072, clone Y69, 1:1000). For signal detection, slides were incubated with ImPRESS horseradish peroxidase secondary antibodies (Dako) and the DAB Peroxidase Substrate Kit (Vector Labs). Identification of primary and metastatic tumors was performed by microscopic examination of H&E-stained and RFP-/GFP-stained sections. Measurements of the percentages of c-Myc were performed using QuPath.

Laser capture microdissection, DNA extraction, and sequencing of tumors:

Tumors identified by IHC were laser-capture microdissected (LCM) from paraffin sections using the Veritas Laser-Capture Microdissection microscope. Briefly, tissues were sectioned into Arcturus PEN Membrane Glass Slides and were H&E stained. Right before LCM, slides were dehydrated by serial incubations with 95% and

100% ethanol, followed by xylene and air dry. Tumors were microdissected as per the manufacturer's instructions. DNA was extracted from individual tumors using the Arcturus PicoPure DNA Extraction Kit (Applied Biosystems). Briefly, tumor sections were incubated in proteinase K extraction solution at 65°C for 16-18 hours, spun down, incubated at 95°C for 10 minutes, and cooled. The Fbxw7 loci was amplified using the primers below and analyzed via Sanger Sequencing followed by TIDE analysis and/or CRISPR sequencing.

Name	Forward primer	Reverse primer
sgFbxw7α	GAACTGCTCTCTGTGGGCA	TGCTCTTCCTGATTTCCCGA
sgFbxw7β	TGGTGCTGGGGAGTTTTGCT	GCACAGGAAGAAAACAGATT
sgFbxw7γ	TGCCTGTAGTACATATTGAGAGTGT	ACAGACAACACACAGGCGAT
sgFbxw7.1	GTGTAGTCTTTGACTTACTT	GTAATGCGAGCCCCAATCAT
sgFbxw7.2	TCTTACGTATAAGCAGGAAATCCA	TCTGTACTCCCACCCTGACC

Tail Vein Injections:

Cells were dissociated to single cells by Trypsin-EDTA (0.25%, Invitrogen) incubation for 5-10 minutes at 37°C, counted using a hemocytometer, and the desired number of cells were resuspended in 1X PBS (100,000 cells/100μL/mouse). Prior to injection, mice were warmed for 5-10 minutes using an overhead heat lamp to dilate the veins and then placed in a tail access rodent restrainer (Stoelting, catalog no. 51338). Lateral tail veins were further dilated with 70% ethanol wipes and cells were injected into the tail vein using a 30-gauge needle. If resistance was encountered and/or a white blister appeared, the needle was removed and reinserted above the first site.

IncuCyte Proliferation, Scratch Wound Healing Assay and Organoid modules:

Cells were plated in 48 well plates (Corning 3338) at a seeding density of ~30,000 cells/well and growth was analyzed using the IncuCyte live-cell imaging analysis system, as per manufacturer's instructions. For scratch wound healing assay, after cells arrived at 80% confluency, a scratch was made using a p20 pipette tip, pictures were taken at different time points using the IncuCyte live-cell imaging system and wound closure was determined using ImageJ. For organoid growth assays, organoids were dissociated as described above, 5,000 cells were plated in 10μL 100% Matrigel domes in 48 well plates (Corning 3338). Analysis was done as per manufacturer's instructions using the IncuCyte Organoid Module which tracks total organoid area, organoid count, darkness, and eccentricity.

Longitudinal circulating tumor cell (CTC) detection:

All animal based procedures were approved by the Massachusetts Institute of Technology Committee of Animal Care (CAC), Division of Comparative Medicine (DCM). Briefly, mice underwent cannulation surgery for circulation through an optical detection platform for CTC detection as previously described on (Hamza et al., 2019). Briefly, candidate mice for the arteriovenous shunt surgery were identified by ultrasound using the Vevo 3100 LAZR-X (FUJIFILM-Visualsonics). Catheters are inserted into the right jugular vein and the left carotid artery and are externalized using standard cannulation surgical techniques in anesthetized mice. A peristaltic pump (Instech Laboratories Inc., Plymoth Meeting, PA, USA) is then connected to the catheters for blood sampling and return through the carotid artery and jugular vein, respectively, in the conscious mouse.

The blood is directed into the main flow channel of the CTC sorter chip. For TdTomato-positive cells, a green (532-nm) laser illuminates two points along the main flow channel of the CTC chip separated by a known distance, and are detected by a photomultiplier tube.

Tumor digestion and Fluorescence-Activated Cell Sorting:

Tumors were manually minced with razor blades and dissociated for 30 minutes at 37oC in digestion buffer: 1X HBSS (Gibco), 10mM HEPES (Gibco), 1% heat-inactivated FBS, 125 U/mL collagenase IV (Worthington), 40 U/mL DNase I, grade II (Roche), 1 U/mL Dispase (Corning), 0.25 mg/mL Trypsin inhibitor (Sigma-Aldrich). The digested cell mixture was diluted with cold HBSS and passed through a 70μm cell strainer. Single cell suspensions were pelleted at 2000 rpm for 5 minutes and transferred to 96 well U-bottom plates for flow cytometry staining. Surface staining of CD45+, CD31+, CD11b+ and TER119+ was performed in PBS with 1% heat-inactivated FBS on ice for 30 minutes in the dark. Right before sorting, the cell suspension was stained with DAPI (Thermo Fisher) for Live/Dead separation. TdTomato positive cancer cells were sorted by FACS, excluding all cells positive for the previous stains.

Mass Spectrometry:

<u>Reduction, Alkylation and Tryptic Digestion:</u>

Cells were lysed in 8M urea. Proteins were reduced with 10mM dithiothreitol (Sigma) for 1h at 56^{o}C and then alkylated with 20mM iodoacetamide (Sigma) for 1h at

25°C in the dark. Proteins were then digested with modified trypsin (Promega) at an enzyme/substrate ratio of 1:50 in 100mM ammonium bicarbonate, pH 8.9 at 25°C overnight. Trypsin activity was halted by addition of formic acid (99.9%, Sigma) to a final concentration of 5%. Peptides were desalted using Pierce peptide desalting spin columns (cat#89851) per manufacturer's instructions.

TMT labeling

Desalted peptides were dissolved in 100 µL of 100 mM triethylammonium bicarbonate, pH 8.5, and the TMT 6-plex, TMT 10plex or 16-Plex reagents was dissolved in 41 µL of anhydrous acetonitrile. The solution containing peptides were mixed with the appropriate TMT reagent, vortexed and incubated at room temperature for 1 h. Samples labeled with different isotopic TMT reagents were combined and concentrated to completion in a vacuum centrifuge.

Peptide Fractionation:

The TMT labeled peptides were fractionated via Pierce High pH Reveresed-phase peptide fractionation kit (cat#84868) per manufacturer's instruction with the slight modification of 10 fractions instead of 8 fractions. . Each of the fractions were speed-vac to dryness than re-suspended in 0.2% formic acid and run on the LC-MS.

LC-MS/MS:

TMT 6-plex and 10-plex: The TMT labeled tryptic peptides were separated by reverse phase HPLC (Thermo Ultimate 3000) using a Thermo PepMap RSLC C18

column (2um tip, 75umx50cm PN# ES903) over a 100 minute gradient before nanoelectrospray using a Exploris mass spectrometer (Thermo). Solvent A was 0.1% formic acid in water and solvent B was 0.1% formic acid in acetonitrile. The gradient conditions were 1% B (0-10 min at 300nL/min) 1% B (10-15 min, 300 nL/min to 200 nL/min) 1-10% B (15-20 min, 200nL/min), 10-25% B (20-68.4 min, 200nL/min), 25-36 B (68.4-75 min, 200nL/min), 36-80% B (75-75.5 min, 200 nL/min), 80% B (75.5-80 min, 200nL/min), 80-1% B (80-80.1 min, 200nL/min), 1% B (80.1-100 min, 200nL/min).

The mass spectrometer was operated in a data-dependent mode. The parameters for the full scan MS were: resolution of 120,000 across 375-1600 m/z and maximum IT 25 ms. The full MS scan was followed by MS/MS for as many precursor ions in a two second cycle with a NCE of 36, dynamic exclusion of 30 s and resolution of 30,000 for 6-plex and 45,000 for 10-plex.

TMT 16-plex: The TMT labeled tryptic peptides were separated by reverse phase HPLC (Thermo Ultimate 3000) using a Thermo PepMap RSLC C18 column(2um tip, 75umx50cm PN# ES903) over a 140 minute gradient before nanoelectrospray using a Exploris mass spectrometer (Thermo). Solvent A was 0.1% formic acid in water and solvent B was 0.1% formic acid in acetonitrile. The gradient conditions were 1% B (0-10 min at 300nL/min) 1% B (10-15 min, 300 nL/min to 200 nL/min) 1-5% B (15-20 min, 200nL/min), 5-25% B (20-104.8 min, 200nL/min), 25-35 B (104.8-112 min, 200nL/min), 35-80% B (112-115.5 min, 200 nL/min), 80% B (115.5-120 min, 200nL/min), 80-1% B (120-120.1 min, 200nL/min), 1% B (120.1.1-140 min, 200nL/min).

The mass spectrometer was operated in a data-dependent mode. The parameters for the full scan MS were: resolution of 60,000 across 450-1600 m/z and maximum IT 50

ms. The full MS scan was followed by MS/MS for as many precursor ions in a two second cycle with a NCE of 36, dynamic exclusion of 30 s and resolution of 45,000.

Database Search and data analysis:

Raw mass spectral data files (.raw) were searched using Sequest HT in Proteome Discoverer (Thermo). Request search parameters were: 20 ppm mass tolerance for precursor ions; 0.05 Da for fragment ion mass tolerance; 2 missed cleavages of trypsin; fixed modifications were carbamidomethylation of cysteine and TMT modification on the lysines and peptide N-termini; variable modifications were methionine oxidation, methionine loss at the N-terminus of the protein, acetylation of the N-terminus of the protein and also Met-loss plus acetylation of the protein N-terminus. For peptide groups data only PSMs with a Xcorr score greater than 2, isolation interference less than or equal to 30 and a deltaM(ppm) between -3 and 3 were used.

Data analysis was performed using the previously described DEqMS R package for differential protein expression analysis (Zhu *et al.,* 2020).

Acknowledgments

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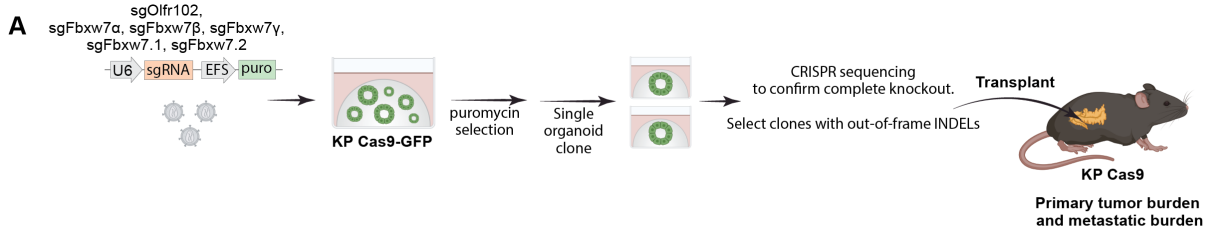
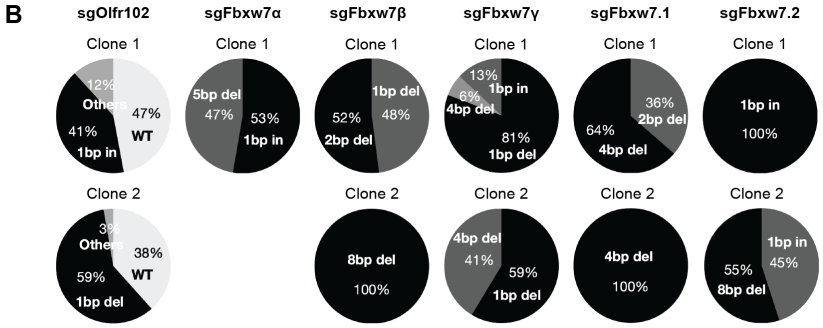
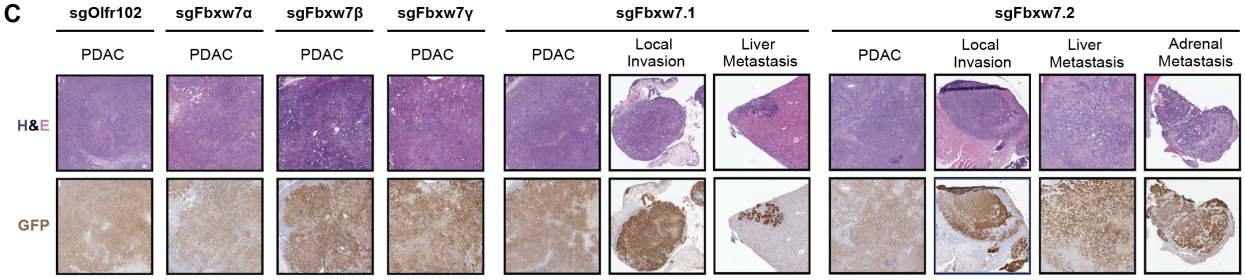
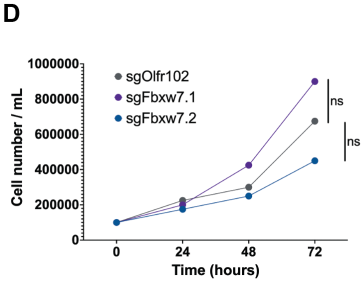
T.J. is a member of the Board of Directors of Amgen and Thermo Fisher Scientific. He is also a co-Founder of Dragonfly Therapeutics and T2 Biosystems. T.J. serves on the Scientific Advisory Board of Dragonfly Therapeutics, SQZ Biotech, and Skyhawk Therapeutics. He is the President of Break Through Cancer. None of these affiliations represent a conflict of interest with respect to the design or execution of this study or interpretation of data presented in this manuscript. T.J. laboratory currently also receives funding from the Johnson & Johnson Lung Cancer Initiative, but this funding did not support the research described in this manuscript.

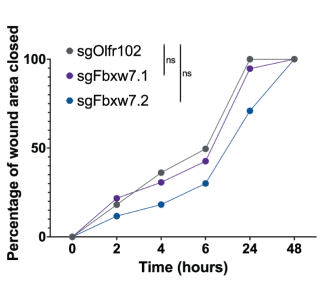
Supplemental Figure 1

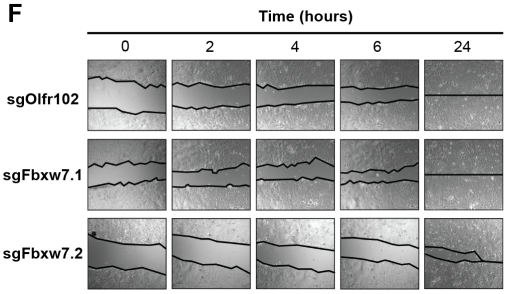












Supplemental Figure 1: Further interrogation of Fbxw7 isoforms in PDAC progression and metastatic potential.

(A) Experimental design. Briefly, KP Cas9-GFP organoids were transducer with lentivirus expressing sgRNAs targeting individual Fbxw7 isoforms (sgFbxw7α, sgFbxw7β and sgFbxw7γ), as well as sgRNA targeting all isoforms at different loci (sgFbxw7.1 and sgFbxw7.2) and control sgOlfr102; and puromycin resistance. Successful transduction was selected by puromycin treatment. Stable organoid lines were single organoid cloned, and editing was confirmed by CRISPR-sequecing. Two clones with out-of-frame INDELs were orthotopically transplanted to syngeneic KP Cas9 mice.

(B) CRISPR-seqeuncing results of selected clones with out-of-frame INDELs at the different Fbxw7 loci. Bp, base pairs; del, deletion; in, insertion; WT, wild type.

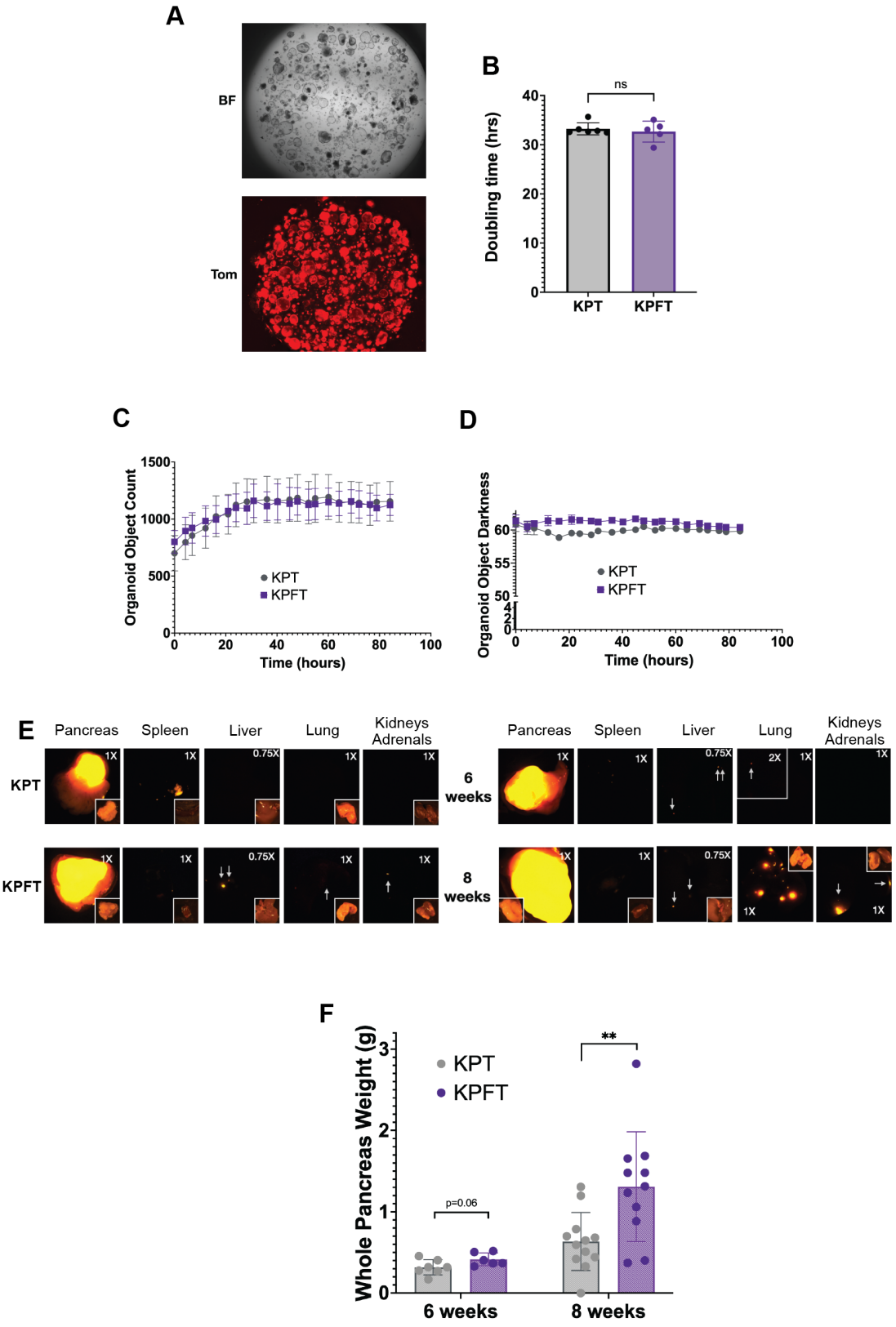
(C) Representative histopathology images of primary and metastatic tumors. GFP immunohistochemistry marks tumors from the transplanted organoids.

(D) In vitro proliferation assay of control organoid line (sgOlfr102) and complete Fbxw7 loss-of-function lines (sgFbxw7.1 and sgFbxw7.2)

(E) Percetange of wound closure after scratch wound healing assay of control organoid line (sgOlfr102) and complete Fbxw7 loss-of-function lines (sgFbxw7.1 and sgFbxw7.2)

(F) Representative images over time of Scratch Wound Healing Assay of control organoid line (sgOlfr102) and complete Fbxw7 loss-of-function lines (sgFbxw7.1 and sgFbxw7.2)

Supplemental Figure 2



Supplemental Figure 2: Further interrogation of the Fbxw7 flexed allele in PDAC progression to metastasis

(A) Representative brightfieqld (BF) and TdTomato fluorescence (Tom) images of derived organoids from KPFT mice.
(B) Doubling time of derived organoids lines KPT and KPFT
(C) Total organoid count over time of KPT and KPFT organoids
(D) Organoid darkness over time of KPT and KPFT organoids as a proxy for organoid necrosis
(E) Representative TdTomato fluorescence images of the pancreas and distal organs show primary tumors and metastatic foci from KPT and KPFT animals at 6 and 8 weeks post transplant. Arrows point at micrometastatic foci.
(F) Whole pancreas weight (grams) as a proxy for primary burden from KPT and KPFT animals at 6 and 8 weeks post transplant.. ** $p<0.01$.

References

Boj, S. F., Hwang, C. I., Baker, L. A., Chio, I. I. C., Engle, D. D., Corbo, V., Jager, M., Ponz-Sarvise, M., Tiriac, H., Spector, M. S., Gracanin, A., Oni, T., Yu, K. H., Van Boxtel, R., Huch, M., Rivera, K. D., Wilson, J. P., Feigin, M. E., Öhlund, D., … Tuveson, D. A. (2015). Organoid models of human and mouse ductal pancreatic cancer. *Cell*, *160*(1–2), 324–338. https://doi.org/10.1016/J.CELL.2014.12.021

Bonetti, P., Davoli, T., Sironi, C., Amati, B., Pelicci, P. G., & Colombo, E. (2008). Nucleophosmin and its AML-associated mutant regulate c-Myc turnover through Fbw7 gamma. *The Journal of Cell Biology*, *182*(1), 19–26. https://doi.org/10.1083/jcb.200711040

Calhoun, E. S., Jones, J. B., Ashfaq, R., Adsay, V., Baker, S. J., Valentine, V., Hempen, P. M., Hilgers, W., Yeo, C. J., Hruban, R. H., & Kern, S. E. (2003). BRAF and FBXW7 (CDC4, FBW7, AGO, SEL10) mutations in distinct subsets of pancreatic cancer: Potential therapeutic targets. *The American Journal of Pathology*, *163*(4), 1255–1260. https://doi.org/10.1016/S0002-9440(10)63485-2

Cenciarelli, C., Chiaur, D. S., Guardavaccaro, D., Parks, W., Vidal, M., & Pagano, M. (1999). Identification of a family of human F-box proteins. *Current Biology*, *9*(20), 1177–1179. https://doi.org/10.1016/S0960-9822(00)80020-2

Ciechanover, A. (2015). The unravelling of the ubiquitin system. *Nature Reviews Molecular Cell Biology*, *16*(5), 322–324. https://doi.org/10.1038/nrm3982

Durgan, J., & Parker, P. J. (2010). Regulation of the tumour suppressor Fbw7α by PKC-dependent phosphorylation and cancer-associated mutations. *The Biochemical Journal*, *432*(1), 77–87. https://doi.org/10.1042/BJ20100799

Fernandez, P. C., Frank, S. R., Wang, L., Schroeder, M., Liu, S., Greene, J., Cocito, A., & Amati, B. (2003). Genomic targets of the human c-Myc protein. *Genes & Development*, *17*(9), 1115–1129. https://doi.org/10.1101/gad.1067003

Foltz, D. R., Santiago, M. C., Berechid, B. E., & Nye, J. S. (2002). Glycogen synthase kinase-3beta modulates notch signaling and stability. *Current Biology: CB*, *12*(12), 1006–1011. https://doi.org/10.1016/s0960-9822(02)00888-6

Forbes, S. A., Beare, D., Boutselakis, H., Bamford, S., Bindal, N., Tate, J., Cole, C. G., Ward, S., Dawson, E., Ponting, L., Stefancsik, R., Harsha, B., Kok, C. Y., Jia, M., Jubb, H., Sondka, Z., Thompson, S., De, T., & Campbell, P. J. (2017). COSMIC: Somatic cancer genetics at high-resolution. *Nucleic Acids Research*, *45*(D1), D777–D783. https://doi.org/10.1093/nar/gkw1121

Gao, J., Azmi, A. S., Aboukameel, A., Kauffman, M., Shacham, S., Abou-Samra, A.-B., & Mohammad, R. M. (2014). Nuclear retention of Fbw7 by specific inhibitors of nuclear export leads to Notch1 degradation in pancreatic cancer. *Oncotarget*, *5*(11), 3444–3454. https://doi.org/10.18632/oncotarget.1813

Grim, J. E., Gustafson, M. P., Hirata, R. K., Hagar, A. C., Swanger, J., Welcker, M., Hwang, H. C., Ericsson, J., Russell, D. W., & Clurman, B. E. (2008). Isoform- and cell

cycle-dependent substrate degradation by the Fbw7 ubiquitin ligase. *The Journal of Cell Biology*, *181*(6), 913–920. https://doi.org/10.1083/jcb.200802076

Gstalder, C., Liu, D., Miao, D., Lutterbach, B., DeVine, A. L., Lin, C., Shettigar, M., Pancholi, P., Buchbinder, E. I., Carter, S. L., Manos, M. P., Rojas-Rudilla, V., Brennick, R., Gjini, E., Chen, P.-H., Lako, A., Rodig, S., Yoon, C. H., Freeman, G. J., … Haq, R. (2020). Inactivation of Fbxw7 Impairs dsRNA Sensing and Confers Resistance to PD-1 Blockade. *Cancer Discovery*, *10*(9), 1296–1311. https://doi.org/10.1158/2159-8290.CD-19-1416

Hamza, B., Ng, S. R., Prakadan, S. M., Delgado, F. F., Chin, C. R., King, E. M., Yang, L. F., Davidson, S. M., DeGouveia, K. L., Cermak, N., Navia, A. W., Winter, P. S., Drake, R. S., Tammela, T., Li, C. M.-C., Papagiannakopoulos, T., Gupta, A. J., Shaw Bagnall, J., Knudsen, S. M., … Manalis, S. R. (2019). Optofluidic real-time cell sorter for longitudinal CTC studies in mouse models of cancer. *Proceedings of the National Academy of Sciences*, *116*(6), 2232–2236. https://doi.org/10.1073/pnas.1814102116

Hao, Z., & Rajewsky, K. (2001). Homeostasis of peripheral B cells in the absence of B cell influx from the bone marrow. *The Journal of Experimental Medicine*, *194*(8), 1151–1164. https://doi.org/10.1084/jem.194.8.1151

He, D., Huang, C., Zhou, Q., Liu, D., Xiong, L., Xiang, H., Ma, G., & Zhang, Z. (2017). HnRNPK/miR-223/FBXW7 feedback cascade promotes pancreatic cancer cell growth and invasion. *Oncotarget*, *8*(12), 20165–20178. https://doi.org/10.18632/oncotarget.15529

Hershko, A., Ciechanover, A., & Rose, I. A. (1979). Resolution of the ATP-dependent proteolytic system from reticulocytes: A component that interacts with ATP. *Proceedings of the National Academy of Sciences of the United States of America*, *76*(7), 3107–3110.

Hingorani, S. R., Petricoin, E. F., Maitra, A., Rajapakse, V., King, C., Jacobetz, M. A., Ross, S., Conrads, T. P., Veenstra, T. D., Hitt, B. A., Kawaguchi, Y., Johann, D., Liotta, L. A., Crawford, H. C., Putt, M. E., Jacks, T., Wright, C. V. E., Hruban, R. H., Lowy, A. M., & Tuveson, D. A. (2003). Preinvasive and invasive ductal pancreatic cancer and its early detection in the mouse. *Cancer Cell*, *4*(6), 437–450. https://doi.org/10.1016/S1535-6108(03)00309-X/ATTACHMENT/E4365F87-FD7D-4C83-B8BD-E8C99A4B7F21/MMC1.PDF

Howlader, N., Noone, A., Krapcho, M., Miller, D., Brest, A., Yu, M., Ruhl, J., Tatalovich, Z., Mariotto, A., Lewis, D., Chen, H., Feuer, E., & Cronin, K. (2021). SEER Cancer Statistics Review, 1975-2018. *SEER*. https://seer.cancer.gov/csr/1975_2018/index.html

Hruban, R. H., Goggins, M., Parsons, J., & Kern, S. E. (2000). Progression model for pancreatic cancer. *Clinical Cancer Research: An Official Journal of the American Association for Cancer Research*, *6*(8), 2969–2972.

Husnjak, K., & Dikic, I. (2012). Ubiquitin-binding proteins: Decoders of ubiquitin-mediated cellular functions. *Annual Review of Biochemistry*, *81*, 291–322. https://doi.org/10.1146/annurev-biochem-051810-094654

Hustinx, S. R., Leoni, L. M., Yeo, C. J., Brown, P. N., Goggins, M., Kern, S. E., Hruban, R. H., & Maitra, A. (2005). Concordant loss of MTAP and p16/CDKN2A expression in pancreatic intraepithelial neoplasia: Evidence of homozygous deletion in a noninvasive precursor lesion. *Modern Pathology: An Official Journal of the United States and Canadian Academy of Pathology, Inc*, *18*(7), 959–963. https://doi.org/10.1038/modpathol.3800377

Inuzuka, H., Tseng, A., Gao, D., Zhai, B., Zhang, Q., Shaik, S., Wan, L., Ang, X. L., Mock, C., Yin, H., Stommel, J. M., Gygi, S., Lahav, G., Asara, J., Xiao, Z.-X. J., Kaelin, W. G., Harper, J. W., & Wei, W. (2010). Phosphorylation by casein kinase I promotes the turnover of the Mdm2 oncoprotein via the SCF(beta-TRCP) ubiquitin ligase. *Cancer Cell*, *18*(2), 147–159. https://doi.org/10.1016/j.ccr.2010.06.015

Ishii, N., Araki, K., Yokobori, T., Gantumur, D., Yamanaka, T., Altan, B., Tsukagoshi, M., Igarashi, T., Watanabe, A., Kubo, N., Hosouchi, Y., Kuwano, H., & Shirabe, K. (2017). Reduced FBXW7 expression in pancreatic cancer correlates with poor prognosis and chemotherapeutic resistance via accumulation of MCL1. *Oncotarget*, *8*(68), 112636–112646. https://doi.org/10.18632/oncotarget.22634

Jackson, E. L., Willis, N., Mercer, K., Bronson, R. T., Crowley, D., Montoya, R., Jacks, T., & Tuveson, D. A. (2001). Analysis of Lung tumor initiationand progression using conditional expression of oncogenic Kras. *Genes and Development*, *617*, 3243–3248. https://doi.org/10.1101/gad.943001.GENES

Ji, S., Qin, Y., Shi, S., Liu, X., Hu, H., Zhou, H., Gao, J., Zhang, B., Xu, W., Liu, J., Liang, D., Liu, L., Liu, C., Long, J., & Zhou, H. (2015). ERK kinase phosphorylates and destabilizes the tumor suppressor FBW7 in pancreatic cancer. *Nature Publishing Group*, 561–573. https://doi.org/10.1038/cr.2015.30

Jin, X., Yang, C., Fan, P., Xiao, J., Zhang, W., Zhan, S., Liu, T., Wang, D., & Wu, H. (2017). CDK5/FBW7-dependent ubiquitination and degradation of EZH2 inhibits pancreatic cancer cell migration and invasion. *Journal of Biological Chemistry*, *292*(15), 6269–6280. https://doi.org/10.1074/jbc.M116.764407

Kanda, M., Matthaei, H., Wu, J., Hong, S., Yu, J., Borges, M., Hruban, R. H., Maitra, A., Kinzler, K., Vogelstein, B., & Goggins, M. (2012). Presence of Somatic Mutations in Most Early-Stage Pancreatic Intraepithelial Neoplasia. *Gastroenterology*, *142*(4), 730-733.e9. https://doi.org/10.1053/j.gastro.2011.12.042

Koepp, D. M., Schaefer, L. K., Ye, X., Keyomarsi, K., Chu, C., Harper, J. W., & Elledge, S. J. (2001). Phosphorylation-dependent ubiquitination of cyclin E by the SCFFbw7 ubiquitin ligase. *Science (New York, N.Y.)*, *294*(5540), 173–177. https://doi.org/10.1126/science.1065203

Kwon, Y.-W., Kim, I.-J., Wu, D., Lu, J., Stock, W. A., Liu, Y., Huang, Y., Kang, H. C., DelRosario, R., Jen, K.-Y., Perez-Losada, J., Wei, G., Balmain, A., & Mao, J.-H. (2012). Pten regulates Aurora-A and cooperates with Fbxw7 in modulating

radiation-induced tumor development. *Molecular Cancer Research: MCR*, *10*(6), 834–844. https://doi.org/10.1158/1541-7786.MCR-12-0025

Lan, H., Tan, M., Zhang, Q., Yang, F., Wang, S., Li, H., Xiong, X., & Sun, Y. (2019). LSD1 destabilizes FBXW7 and abrogates FBXW7 functions independent of its demethylase activity. *Proceedings of the National Academy of Sciences*, *116*(25), 12311–12320. https://doi.org/10.1073/pnas.1902012116

Lee, A. Y. L., Dubois, C. L., Sarai, K., Zarei, S., Schaeffer, D. F., Sander, M., & Kopp, J. L. (2019). Cell of origin affects tumour development and phenotype in pancreatic ductal adenocarcinoma. *Gut*, *68*(3), 487–498. https://doi.org/10.1136/GUTJNL-2017-314426

Li, Y., Hu, K., Xiao, X., Wu, W., Yan, H., Chen, H., Chen, Z., & Yin, D. (2018). FBW7 suppresses cell proliferation and G2/M cell cycle transition via promoting γ-catenin K63-linked ubiquitylation. *Biochemical and Biophysical Research Communications*, *497*(2), 473–479. https://doi.org/10.1016/j.bbrc.2018.01.192

Madisen, L., Zwingman, T. A., Sunkin, S. M., Oh, S. W., Zariwala, H. A., Gu, H., Ng, L. L., Palmiter, R. D., Hawrylycz, M. J., Jones, A. R., Lein, E. S., & Zeng, H. (2010). A robust and high-throughput Cre reporting and characterization system for the whole mouse brain. *Nature Neuroscience*, *13*(1), 133–140. https://doi.org/10.1038/nn.2467

Mao, J.-H., Kim, I.-J., Wu, D., Climent, J., Kang, H. C., DelRosario, R., & Balmain, A. (2008). FBXW7 Targets mTOR for Degradation and Cooperates with PTEN in Tumor Suppression. *Science*, *321*(5895), 1499–1502. https://doi.org/10.1126/science.1162981

Marino, S., Vooijs, M., Van Der Gulden, H., Jonkers, J., & Berns, A. (2000). Induction of medulloblastomas in p53-null mutant mice by somatic inactivation of Rb in the external granular layer cells of the cerebellum. *Genes and Development*, *14*(8), 994–1004. https://doi.org/10.1101/gad.14.8.994

Matsumoto, A., Onoyama, I., & Nakayama, K. I. (2006). Expression of mouse Fbxw7 isoforms is regulated in a cell cycle- or p53-dependent manner. *Biochemical and Biophysical Research Communications*, *350*(1), 114–119. https://doi.org/10.1016/j.bbrc.2006.09.003

Morton, J. P., Timpson, P., Karim, S. A., Ridgway, R. A., Athineos, D., Doyle, B., Jamieson, N. B., Oien, K. A., Lowy, A. M., Brunton, V. G., Frame, M. C., Evans, T. R. J., & Sansom, O. J. (2010). Mutant p53 drives metastasis and overcomes growth arrest/senescence in pancreatic cancer. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(1), 246–251. https://doi.org/10.1073/PNAS.0908428107

Pérez-Mancera, P. A., Rust, A. G., van der Weyden, L., Kristiansen, G., Li, A., Sarver, A. L., Silverstein, K. A. T., Grützmann, R., Aust, D., Rümmele, P., Knösel, T., Herd, C., Stemple, D. L., Kettleborough, R., Brosnan, J. A., Li, A., Morgan, R., Knight, S., Yu, J., … Tuveson, D. A. (2012). The deubiquitinase USP9X suppresses pancreatic ductal adenocarcinoma. *Nature*, *486*(7402), 266–270. https://doi.org/10.1038/nature11114

Popovic, D., Vucic, D., & Dikic, I. (2014). Ubiquitination in disease pathogenesis and treatment. *Nature Medicine*, *20*(11), 1242–1253. https://doi.org/10.1038/nm.3739

Qi, J., & Ronai, Z. A. (2015). Dysregulation of ubiquitin ligases in cancer. *Drug Resistance Updates: Reviews and Commentaries in Antimicrobial and Anticancer Chemotherapy*, *23*, 1–11. https://doi.org/10.1016/j.drup.2015.09.001

Ryan, D. P., Hong, T. S., & Bardeesy, N. (2014). Pancreatic Adenocarcinoma. *New England Journal of Medicine*, *371*(11), 1039–1049. https://doi.org/10.1056/NEJMra1404198

Sancho, R., Gruber, R., Gu, G., & Behrens, A. (2014). Loss of Fbw7 Reprograms Adult Pancreatic Ductal Cells into α, δ, and β Cells. *Cell Stem Cell*, *15*(2), 139–153. https://doi.org/10.1016/j.stem.2014.06.019

Sastra, S. A., & Olive, K. P. (2013). Quantification of murine pancreatic tumors by high-resolution ultrasound. *Methods in Molecular Biology (Clifton, N.J.)*, *980*, 249–266. https://doi.org/10.1007/978-1-62703-287-2_13

Senft, D., Qi, J., & Ronai, Z. A. (2018). Ubiquitin ligases in oncogenic transformation and cancer therapy. *Nature Reviews Cancer*, *18*(2), 69–88. https://doi.org/10.1038/nrc.2017.105

Skaar, J. R., Pagan, J. K., & Pagano, M. (2014). SCF ubiquitin ligase-targeted therapies. *Nature Reviews. Drug Discovery*, *13*(12), 889–903. https://doi.org/10.1038/nrd4432

Song, Y., Lai, L., Chong, Z., He, J., Zhang, Y., Xue, Y., Xie, Y., Chen, S., Dong, P., Chen, L., Chen, Z., Dai, F., Wan, X., Xiao, P., Cao, X., Liu, Y., & Wang, Q. (2017). E3 ligase FBXW7 is critical for RIG-I stabilization during antiviral responses. *Nature Communications*, *8*(1), 14654. https://doi.org/10.1038/ncomms14654

Spruck, C. H., Strohmaier, H., Sangfelt, O., Müller, H. M., Hubalek, M., Müller-Holzner, E., Marth, C., Widschwendter, M., & Reed, S. I. (2002). HCDC4 gene mutations in endometrial cancer. *Cancer Research*, *62*(16), 4535–4539.

Thompson, B. J., Jankovic, V., Gao, J., Buonamici, S., Vest, A., Lee, J. M., Zavadil, J., Nimer, S. D., & Aifantis, I. (2008). Control of hematopoietic stem cell quiescence by the E3 ubiquitin ligase Fbw7. *The Journal of Experimental Medicine*, *205*(6), 1395–1408. https://doi.org/10.1084/jem.20080277

Uhlén, M., Björling, E., Agaton, C., Szigyarto, C. A.-K., Amini, B., Andersen, E., Andersson, A.-C., Angelidou, P., Asplund, A., Asplund, C., Berglund, L., Bergström, K., Brumer, H., Cerjan, D., Ekström, M., Elobeid, A., Eriksson, C., Fagerberg, L., Falk, R., … Pontén, F. (2005). A Human Protein Atlas for Normal and Cancer Tissues Based on Antibody Proteomics. *Molecular & Cellular Proteomics*, *4*(12), 1920–1932. https://doi.org/10.1074/mcp.M500279-MCP200

Wei, W., Jin, J., Schlisio, S., Harper, J. W., & Kaelin, W. G. (2005). The v-Jun point mutation allows c-Jun to escape GSK3-dependent recognition and destruction by the Fbw7 ubiquitin ligase. *Cancer Cell*, *8*(1), 25–33. https://doi.org/10.1016/j.ccr.2005.06.005

Welcker, M., Orian, A., Grim, J. E., Grim, J. A., Eisenman, R. N., & Clurman, B. E. (2004). A nucleolar isoform of the Fbw7 ubiquitin ligase regulates c-Myc and cell size. *Current Biology: CB*, *14*(20), 1852–1857. https://doi.org/10.1016/j.cub.2004.09.083

Wilentz, R. E., Iacobuzio-Donahue, C. A., Argani, P., McCarthy, D. M., Parsons, J. L., Yeo, C. J., Kern, S. E., & Hruban, R. H. (2000). Loss of expression of Dpc4 in pancreatic intraepithelial neoplasia: Evidence that DPC4 inactivation occurs late in neoplastic progression. *Cancer Research*, *60*(7), 2002–2006.

Winston, J. T., Koepp, D. M., Zhu, C., Elledge, S. J., & Harper, J. W. (1999). A family of mammalian F-box proteins. *Current Biology*, *9*(20), 1180–1182. https://doi.org/10.1016/S0960-9822(00)80021-4

Yada, M., Hatakeyama, S., Kamura, T., Nishiyama, M., Tsunematsu, R., Imaki, H., Ishida, N., Okumura, F., Nakayama, K., & Nakayama, K. I. (2004). Phosphorylation-dependent degradation of c-Myc is mediated by the F-box protein Fbw7. *The EMBO Journal*, *23*(10), 2116–2125. https://doi.org/10.1038/sj.emboj.7600217

Yau, R., & Rape, M. (2016). The increasing complexity of the ubiquitin code. *Nature Cell Biology*, *18*(6), 579–586. https://doi.org/10.1038/ncb3358

Ye, Z., Zhuo, Q., Hu, Q., Xu, X., Mengqi liu, Zhang, Z., Xu, W., Liu, W., Fan, G., Qin, Y., Yu, X., & Ji, S. (2021). FBW7-NRA41-SCD1 axis synchronously regulates apoptosis and ferroptosis in pancreatic cancer cells. *Redox Biology*, *38*, 101807. https://doi.org/10.1016/j.redox.2020.101807

Yeh, C. H., Bellon, M., & Nicot, C. (2018). FBXW7: A critical tumor suppressor of human cancers. *Molecular Cancer*, *17*(1), 1–19. https://doi.org/10.1186/s12943-018-0857-2

Yumimoto, K., & Nakayama, K. I. (2020). *Recent insight into the role of FBXW7 as a tumor suppressor—ClinicalKey*. https://www-clinicalkey-com.ezp-prod1.hul.harvard.edu/#!/content/playContent/1-s2.0-S1044579X2030050X?scrollTo=%23bib0030

Zhang, Q., Karnak, D., Tan, M., Lawrence, T. S., Morgan, M. A., & Sun, Y. (2016). FBXW7 Facilitates Nonhomologous End-Joining via K63-Linked Polyubiquitylation of XRCC4. *Molecular Cell*, *61*(3), 419–433. https://doi.org/10.1016/j.molcel.2015.12.010

Zhang, Q., Zhang, Y., Parsels, J. D., Lohse, I., Lawrence, T. S., Pasca di Magliano, M., Sun, Y., & Morgan, M. A. (2016). Fbxw7 Deletion Accelerates KrasG12D-Driven Pancreatic Tumorigenesis via Yap Accumulation. *Neoplasia (United States)*, *18*(11), 666–673. https://doi.org/10.1016/j.neo.2016.08.009

CHAPTER 3

DISCUSSION AND FUTURE DIRECTIONS

Discussion and Future Directions

The genetic progression of pancreatic cancer from a normal cell to pre-invasive lesions (PanINs) and finally to PDAC is well understood. However, the molecular processes behind the metastatic spread of PDAC remain unclear. Therefore, there is still a need to discover novel modulators of PDAC metastasis for better treatments for this devastating disease.

Advances in this field have been made by several groups who have identified some novel genetic and epigenetic regulators of PDAC. As covered in Chapter 1, there have been a few studies that have evaluated naive primary tumor and metastases heterogeneity through whole-genome sequencing such as Yachida *et al* (2010), Campbell *et al* (2010), Sanborn *et al* (2015), Hoogstraat *et al* (2014) and Makohon-Moore *et al* (2018). However, studies have not yet identified a recurrent, metastasis-specific driver mutation. Furthermore, acquired molecular changes can promote the spread of primary tumor cells to distant tissues. Chiou *et al* (2017) used genetically engineered mouse models (GEMMs) of PDAC and uncovered a transient subpopulation of cancer cells with exceptionally high metastatic potential driven by the transcription factor BLIMP1. In addition, Whittle *et al* (2015) identified that the transcription factor RUNX3 controls a metastatic switch in PDAC using GEMMs. Lastly, Maddipati *et al* (2021) used a multi-fluorescent lineage labeled mouse model of PDAC to examine primary tumors and matched metastases in order to gauge the degree of metastatic heterogeneity in the tumor-bearing mice, which was connected to higher levels of Myc expression. Moreover, studies have identified epigenetic modulators of PDAC metastasis. Roe *et al* (2017) shows that metastatic progression of PDAC involves large-

scale enhancer reprogramming by FOXA1, which activates an early endodermal stem cell transcriptional program.

Despite the clinical importance of metastatic spread, our knowledge of the molecular processes that underlie PDAC's capacity for metastatic spread is still limited, which prompted me to question metastatic progression at the protein abundance level. The work presented in this thesis focuses on gaining a deeper understanding of the dynamics of pancreatic ductal adenocarcinoma (PDAC) progression to metastasis in the context of FBXW7 dysregulation (Figure 1). I have demonstrated that *FBXW7* loss accelerates metastatic progression of PDAC in the KP (*Kras*$^{LSL\text{-}G12D/+}$; *Trp53*$^{fl/fl}$) mouse model in both immunocompromised and immunocompetent hosts. I showed that only complete loss-of-function of all *FBXW7* isoforms leads to accelerated metastatic progression of PDAC, while isoforms can compensate for the loss of any one of them. Interestingly, I showed that the phenotype is rescued by re-expression of wild type *FBXW7*, while the expression of the hotspot mutant FBXW7^{R465C} recapitulates the phenotype seen in complete loss-of-function of *FBXW7*. Lastly, early efforts to mechanistically interrogate the role of *FBXW7* in PDAC progression to metastases showed a myriad of dysregulated pathways including c-Myc targets, the unfolded protein response, hydrogen peroxide catabolic pathways, and the anti-tumor immune response, among others. These findings deepen our understanding of the biology behind pancreatic cancer progression to metastasis. In this section, I will discuss the implications of these findings for our understanding of pancreatic cancer biology, metastatic progression, and the potential clinical translatability that further work could reveal.

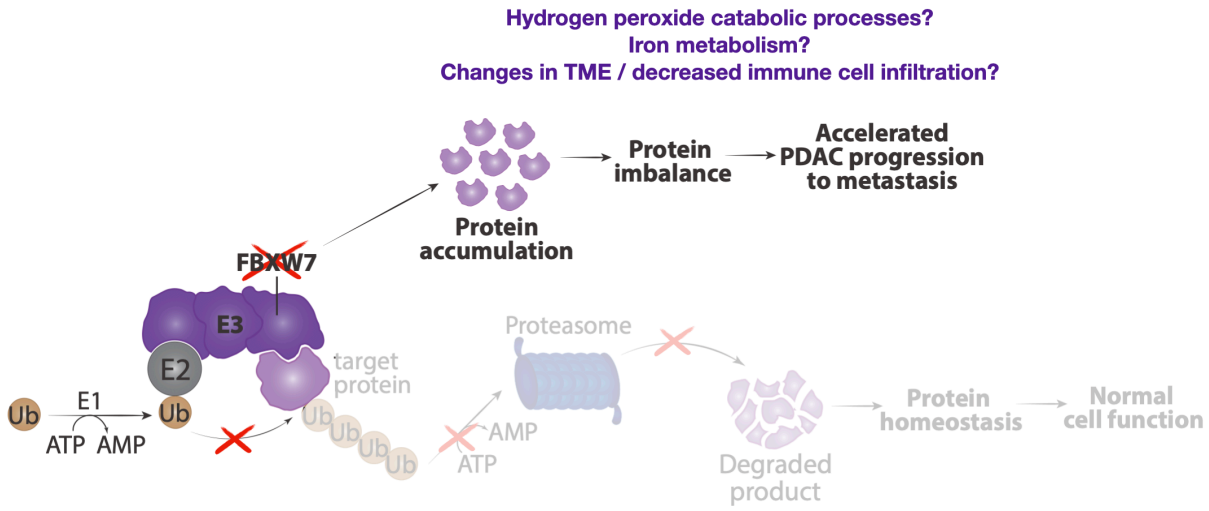


Figure 1: Working model presented in this thesis. In normal cells, FBXW7 recognizes substrates and catalyzes polyubiquitination of the substrate which is then recognized and degraded by the proteasome. This maintains protein homeostasis and normal cell function. When FBXW7 function is lost, substrates are no longer polyubiquitinated and degraded, causing their accumulation leading to protein imbalance and accelerated PDAC progression to metastasis.

3.1 FBXW7 role in PDAC metastasis remains largely understudied

FBXW7 is mutated in ~3.8% of pancreatic cancer patients, and low expression of FBXW7 has been associated with a poor probability of survival compared to patients with high FBXW7 expression (Figure 13, 15). Collectively, this supports that FBXW7 behaves as a tumor suppressor in PDAC.

Since the mutational frequency of FBXW7 in pancreatic cancer is not high, a frequent question that I ask myself is: what other mechanisms are at play in PDAC that lead to loss-of-function of FBXW7? This has been studied to some extent in other cancer types such as glioblastoma, breast cancer, colorectal cancer, esophageal cancer and leukemia (Kim et al., 2012; Li et al., 2014; Ma et al., 2016; Mansour et al., 2013; Teplyuk et al., 2015; Yokobori et al., 2012). However, not many studies have

looked at other mechanisms of FBXW7 dysregulation in PDAC. There is evidence that ERK kinase phosphorylates and destabilizes FBXW7 in pancreatic cancer (Ji *et al.,* 2015). In this work, the scientists discovered that, predominantly as a result of KRAS mutations in pancreatic cancer, low FBXW7 expression was correlated with ERK activation in clinical samples. They also demonstrated that FBXW7 was directly contacted by ERK, which phosphorylates FBXW7 at Thr205, promoting FBXW7 ubiquitination and proteasomal degradation. These findings showed how the oncogenic KRAS mutation suppresses the tumor suppressor FBXW7, highlighting the pivotal role that oncogenic KRAS play in accelerating pancreatic cancer progression. Nevertheless, the aforementioned studies have revealed other epigenetic, post-transcriptional and post-translational mechanisms of FBXW7 dysregulation in cancer that are worth further exploring in PDAC.

Moreover, as I covered in chapter 2, FBXW7 loss-of-function significantly accelerates PDAC progression to metastasis, revealing that FBXW7 is an important tumor suppressor in PDAC. However, just looking at its mutational frequency in PDAC would not necessarily suggest the same. Since FBXW7 dysregulation could also happen post-transcriptionally and post-translationally, it raises the importance of all the efforts to map the proteome in health and disease, such as the Human Protein Atlas (Uhlén et al., 2005, 2015). The proteome is the chief mediator of cellular function, therefore, a better understanding of how protein abundance and stability change from normal tissues to different diseases has the potential to uncover novel and druggable targets for better therapeutics. Furthermore, having matched samples from primary and metastatic tumors could significantly improve our understanding of metastatic drivers.

This work also revealed interesting FBXW7 biology in PDAC. In chapter 1, I covered the molecular characteristics of FBXW7. Briefly, FBXW7 has three functional domains: a dimerization domain (DD), an F-box domain which interacts with the rest of the SCF E3 complex, and eight WD40 repeats which interact with the different substrates (Sprunk *et al.,* 2002; Yeh et al., 2018). There are three FBXW7 isoforms: FBXW7α, FBXW7β, and FBXW7γ, which only differ in their N-terminal signals, sending them to different cellular compartments, further restricting the substrate pool they can sample (Sprunk *et al.,* 2002; Yeh et al., 2018). The most common FBXW7 mutations are found in mutational hotspots, R505, R465, and R479, codons located in the WD40 substrate binding domains, which may reduce its ability to form a stable SCF complex or may affect its interaction with specific substrates (Forbes et al., 2017; Yeh et al., 2018). Interestingly, in chapter 2 I showed that in the KP mouse model of PDAC, different FBXW7 variants have different effects on tumor progression. Firstly, I showed that the loss of an individual Fbxw7 isoform does not accelerate metastatic progression in PDAC mouse models. This suggests that the other functional isoforms are able to restore the function of the lost isoform. An interesting question to be further explored is the mechanisms behind this phenomenon. Can the different FBXW7 isoforms translocate to other subcellular compartments to compensate for the lost isoform, or are they able to restore the lost isoform since they all share the same functional domains? These are questions that I continue to ask myself. Furthermore, I showed in chapter 2 that the hotspot mutant FBXW7^{R465C} recapitulates the phenotype seen in complete loss-of-function of FBXW7, while FBXW7^{R505C} does not. Is this a human to mouse difference? Do these mutants affect differently the stability of the

protein? Since these mutants are in different WD40 repeats, are they affecting different substrates with distance relevance to the disease in our mouse models? These are very intriguing questions, and very interesting question to further pursue. More interestingly, deletion of the WD40 domains only partially recapitulates complete loss-of-function phenotype, which suggests that FBXW7 plays other functions in PDAC progression in addition to supplying substrates for polyubiquitination and degradation. In fact, non-canonical functions of *FBXW7* including complex formation, target protein stabilization, DNA damage repair and cell proliferation, have been previously described (Lan et al., 2019; Li et al., 2018; Song et al., 2017, p. 2; Zhang, Karnak, et al., 2016).

In addition, the DOX inducible system developed in this study allows us to re-express different FBXW7 functional versions once a tumor is established and dependent on the loss of FBXW7. This will give great insights into the role of FBXW7 in PDAC tumor progression, which has not been done in the literature to this point. To achieve this, we need the re-expression to be very tight and controlled. An improvement to this system would be to add a degron domain to either the inner of interest or the rtTA itself, such that it is degraded under normal conditions, and only in the present of TMP (trimethoprim) would the system be stabilized. Therefore, in order to re-express the insert of interest, both DOX and TMP need to be present, allowing more control to the system. Future studies using these models could further dissect the different functional roles of *FBXW7* in PDAC progression, which could also be related to the fact that the majority of the differentially expressed proteins identified in the proteomics studies presented in this thesis are not known or predicted FBXW7 targets.

3.2 Proteomics is a powerful tool to better understand global changes in protein abundance, but still poses limitations

To better understand the mechanism of action by which loss of FBXW7 leads to accelerated progression of PDAC, Chapter 2 describes the use of global proteomics of murine tumors when FBXW7 is lost. These studies revealed the dysregulation of multiple pathways, including: an enrichment of c-Myc targets, hypoxia response pathways, the unfolded protein response, hydrogen peroxide catabolic processes and glutathione catabolic processes, among others. Obvious future direction for this project is the dissection of these pathways and how they play a role in FBXW7 biology in PDAC progression. However, because FBXW7 targets many proteins involved in the regulation of a myriad of cellular processes important in cancer progression, there is a high probability that it is not just one pathway in play, but rather the combination of many of these dysregulated pathways that are leading to the accelerated metastatic progression of PDAC when FBXW7 is lost. This makes the task of finding the mechanism of action extremely challenging. A passion of mine is to find vulnerabilities to treat these tumors and find better therapeutics for PDAC patients, but, this is equally challenging for the same reasons. In an attempt to find vulnerabilities of KPFT tumors, I treated KPT and KPFT organoids with an inhibitor of myeloperoxidase (MPOi), involved in hydrogen peroxide catabolic processes, and iron chelators, two pathways seen consistently enriched in KPFT tumors, and no significant difference in organoids growth and death was observed (Figure 2). However, an unbiased CRISPR screen could be a good strategy to tackle this question with more depth. To take this idea a step further, it would be very interesting to look at cooperation between the different dysregulated

pathways. For this, a CRISPR screen using the endonuclease Cpf1 will be a great strategy. Cpf1 has the ability to process its own CRISPR ran (crRNA) from the same transcript, which allows simple and efficient multiplexed genome editing. This has the potential to find synergistic effects of targeting different identified pathways, which could uncover potential novel combination therapies.

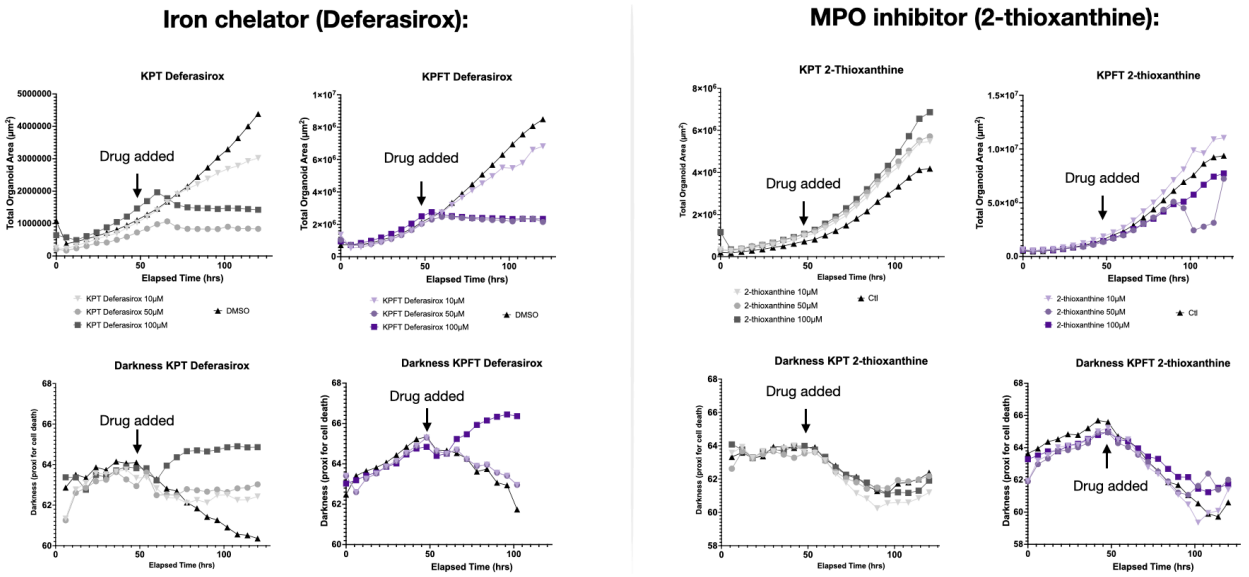


Figure 2: Iron chelators and MPO inhibitor treatments of KPT and KPFT organoids. Organoids were exposed to increasing concentrations of the inhibitors and organoid growth was measured longitudinally using live-imaging and quantification tools of the IncuCyte.

A surprising result consistently observed in this work is the downregulation of inflammatory processes such as interferon alpha and gamma response and antigen processing and presentation. These dysregulated pathways raise interesting biology of *FBXW7* in pancreatic cancer to be further explored. Recent studies have shown that FBXW7 loss impairs dsRNA sensing pathways leading to impaired production of type I interferons and altered tumor microenvironment (Gstalder et al., 2020; Song et al., 2017). In more details, these studies show that FBXW7 plays an important role in

antiviral immune responses by maintaining the stability of RIG-I, meaning that FBXW7 protects RIG-I from degradation. Therefore, inactivation of FBXW7, leads to unstable RIG-I which impairs the dsRNA sensing pathway leading to impaired immune responses. This further supports the hypothesis that FBXW7 plays roles in PDAC progression to metastasis, outside of its main function as a substrate recognition component of the SCF E3 ubiquitin ligase. Interestingly, these studies also show that FBXW7 inactivation leads to resistance to the immunotherapy PD-1 blockade (Gstalder et al., 2020; Song et al., 2017). This is particularly important in PDAC since PDAC is known to be largely unresponsive to immunotherapies (Hilmi et al., 2018). Therefore, a better understanding of novel mechanisms of treatment resistance can help identify patients that would benefit from these treatments, as well as potentially identify novel therapeutic targets for these patients. In an effort to begin the study of changes in the tumor microenvironment when FBXW7 is lost in PDAC, I looked at overall changes in immune cell infiltration in tumors by immunohistochemistry and flow cytometry analysis. Immunohistochemistry against CD4+ T cells, CD8+ T cells, and regulatory T cells (Tregs) in KPT and KPFT tumors revealed an overall decrease in tumor immune cell infiltration when FBXW7 is lost (Figure 3A). Further analysis using flow cytometry revealed that there is also a significant decrease in antigen-experienced CD4+ T cells, CD8+ T cells, as well as a significant decrease in overall and antigen-experienced Tregs (Figure 3B). Collectively, my hypothesis is that loss of FBXW7 cannot stabilize the RIG-I sensing pathway, leading to a decrease in type I interferons and pro-inflammatory cytokines which are needed to activate an immune response again the cancer cells.

Because there's not an active immune response to begin with, Tregs are not as needed in the tumor microenvironment to dampen the immune response.

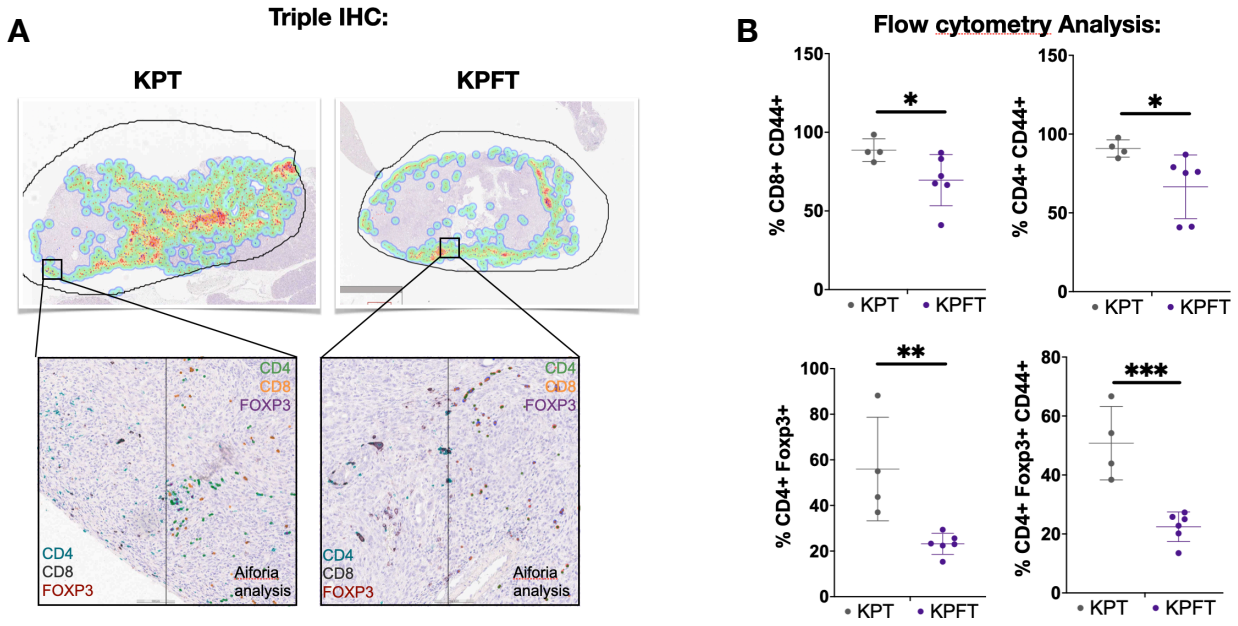


Figure 3: Changes in the tumor microenvironment when FBXW7 is lost. (A) Representative images of KPT and KPFT tumors after triple Immunohistochemistry against CD4+ T cells, CD8+ T cells and regulatory T cells (Tregs). Heatmap created with Aiforia software. **(B)** Quantification of flow cytometry analysis of immune cells in the tumor microenvironment of KPT and KPFT tumors. Graphs show the percentage of CD45+ cells that are antigen experienced (CD44+) CD4+ T cells, CD8+ T cells and regulatory T cells (Tregs). *p<0.05; **p<0.01, ***p<0.001.

However, further studies are needed to better understand this mechanism. With this versatile system in place we could further investigate the changes in the tumor microenvironment when FBXW7 is lost. Firstly, introducing a defined antigen, such as the MHC-I restricted antigens: $OVA_{257–264}$ [SIINFEKL], LAMA4-G1254V or ALG8-A506T (Alspach et al., 2019; Gubin et al., 2014; Freed-Pastor et al., 2021) to the tumors will allow us to better track tumor specific immune response in the context of FBXW7 knockout. In addition, looking at changes in the myeloid compartment both in the tumor microenvironment and the draining lymph nodes could also raise very interesting

biology in this setting, since the literature points that FBXW7 affects early activation mechanisms, therefore we would expect changes in dendritic cells function. Lastly, our data also shows that antigen processing and presentation pathways are downregulated in the FBXW7 knockout setting, therefore, a closer look at the different members in this pathway as well as the differences in antigens being presented specifically in the tumor cells could further our understanding of FBXW7 role in the tumor microenvironment.

While the proteomics data revealed known and novel biology of FBXW7 loss in PDAC tumors, the technique has some limitations, one of which is the need for large amounts of protein material in order to obtain good coverage data. This was particularly challenging in this study because PDAC tumors are known to have low purity, meaning only ~10-20% of the tumors are actually cancer cells. For the study of primary tumors, we were still able to obtain enough material to generate this rich dataset. However, I was particularly interested in comparing the proteome of matched primary and metastatic tumors in both KPT and KPFT tumors. However, these animals succumb to the primary tumor burden before the metastatic lesions are significantly large to obtain enough material for proteomics studies. Unfortunately, we were not able to do this with the techniques available to us at this time. This raises the importance of continuing work in the development and improvement of novel proteomics technologies that have the ability to effectively detect proteins in low-input samples. Some recent technologies, such as the development of single-cell mass spectrometry (Tajik et al., 2022) have great potential to improve these issues, and therefore, we should consider pursuing them for this and other projects.

3.3 Final Perspectives

Translating basic cancer research findings into therapies is a lengthy process. Similar to the history of the development of molecular targeted therapies directed at oncogenes such as KRAS, EGFR, and BRAF, targeting tumor suppressor genes is a decade-long effort. Therefore, continued progress in basic research of tumor suppressor genes biology and related fields such as signaling pathways, the cancer epigenome, and the immune system, are essential to inform translational laboratory work bringing novel compounds to the clinic. In this thesis, we present for the first time the molecular consequences of the loss of the tumor suppressor FBXW7 in the KP (*Kras*$^{LSL\text{-}G12D/+}$; *Trp53*$^{fl/fl}$) mouse model of pancreatic ductal adenocarcinoma (PDAC), as well as the different FBXW7 hotspot mutants found in cancers. This study shows great potential to identify FBXW7-specific vulnerabilities for better therapeutics.

References

Alspach, E., Lussier, D. M., Miceli, A. P., Kizhvatov, I., DuPage, M., Luoma, A. M., Meng, W., Lichti, C. F., Esaulova, E., Vomund, A. N., Runci, D., Ward, J. P., Gubin, M. M., Medrano, R. F. V., Arthur, C. D., White, J. M., Sheehan, K. C. F., Chen, A., Wucherpfennig, K. W., … Schreiber, R. D. (2019). MHC-II neoantigens shape tumour immunity and response to immunotherapy. Nature, 574(7780), 696–701. https://doi.org/10.1038/s41586-019-1671-8

Campbell, P. J., Yachida, S., Mudie, L. J., Stephens, P. J., Pleasance, E. D., Stebbings, L. A., Morsberger, L. A., Latimer, C., McLaren, S., Lin, M. L., McBride, D. J., Varela, I., Nik-Zainal, S. A., Leroy, C., Jia, M., Menzies, A., Butler, A. P., Teague, J. W., Griffin, C. A., … Futreal, P. A. (2010). The patterns and dynamics of genomic instability in metastatic pancreatic cancer. Nature, 467(7319), 1109–1113. https://doi.org/10.1038/nature09460

Chiou, S. H., Risca, V. I., Wang, G. X., Yang, D., Gruner, B. M., Kathiria, A. S., Ma, R. K., Vaka, D., Chu, P., Kozak, M., Castellini, L., Graves, E. E., Kim, G. E., Mourrain, P., Koong, A. C., Giaccia, A. J., & Winslow, M. M. (2017). BLIMP1 induces transient metastatic heterogeneity in pancreatic cancer. Cancer Discovery, 7(10), 1184–1199. https://doi.org/10.1158/2159-8290.CD-17-0250

Freed-Pastor, W. A., Lambert, L. J., Ely, Z. A., Pattada, N. B., Bhutkar, A., Eng, G., Mercer, K. L., Garcia, A. P., Lin, L., Rideout, W. M., Hwang, W. L., Schenkel, J. M., Jaeger, A. M., Bronson, R. T., Westcott, P. M. K., Hether, T. D., Divakar, P., Reeves, J. W., Deshpande, V., … Jacks, T. (2021). The CD155/TIGIT axis promotes and maintains immune evasion in neoantigen-expressing pancreatic cancer. Cancer Cell, 39(10), 1342-1360.e14. https://doi.org/10.1016/j.ccell.2021.07.007

Gstalder, C., Liu, D., Miao, D., Lutterbach, B., DeVine, A. L., Lin, C., Shettigar, M., Pancholi, P., Buchbinder, E. I., Carter, S. L., Manos, M. P., Rojas-Rudilla, V., Brennick, R., Gjini, E., Chen, P.-H., Lako, A., Rodig, S., Yoon, C. H., Freeman, G. J., … Haq, R. (2020). Inactivation of Fbxw7 Impairs dsRNA Sensing and Confers Resistance to PD-1 Blockade. Cancer Discovery, 10(9), 1296–1311. https://doi.org/10.1158/2159-8290.CD-19-1416

Gubin, M. M., Zhang, X., Schuster, H., Caron, E., Ward, J. P., Noguchi, T., Ivanova, Y., Hundal, J., Arthur, C. D., Krebber, W.-J., Mulder, G. E., Toebes, M., Vesely, M. D., Lam, S. S. K., Korman, A. J., Allison, J. P., Freeman, G. J., Sharpe, A. H., Pearce, E. L., … Schreiber, R. D. (2014). Checkpoint blockade cancer immunotherapy targets tumour-specific mutant antigens. Nature, 515(7528), 577–581. https://doi.org/10.1038/nature13988

Hilmi, M., Bartholin, L., & Neuzillet, C. (2018). Immune therapies in pancreatic ductal adenocarcinoma: Where are we now? World Journal of Gastroenterology, 24(20), 2137–2151. https://doi.org/10.3748/wjg.v24.i20.2137

Hoogstraat, M., de Pagter, M. S., Cirkel, G. A., van Roosmalen, M. J., Harkins, T. T., Duran, K., Kreeftmeijer, J., Renkens, I., Witteveen, P. O., Lee, C. C., Nijman, I. J., Guy, T., van 't Slot, R., Jonges, T. N., Lolkema, M. P., Koudijs, M. J., Zweemer, R. P., Voest, E. E., Cuppen, E., & Kloosterman, W. P. (2014). Genomic and

transcriptomic plasticity in treatment-naive ovarian cancer. Genome Research, 24(2), 200–211. https://doi.org/10.1101/gr.161026.113

Ji, S., Qin, Y., Shi, S., Liu, X., Hu, H., Zhou, H., Gao, J., Zhang, B., Xu, W., Liu, J., Liang, D., Liu, L., Liu, C., Long, J., & Zhou, H. (2015). ERK kinase phosphorylates and destabilizes the tumor suppressor FBW7 in pancreatic cancer. Nature Publishing Group, 561–573. https://doi.org/10.1038/cr.2015.30

Kim, H. S., Woolard, K., Lai, C., Bauer, P. O., Maric, D., Song, H., Li, A., Kotliarova, S., Zhang, W., & Fine, H. A. (2012). Gliomagenesis arising from Pten- and Ink4a/Arf-deficient neural progenitor cells is mediated by the p53-Fbxw7/Cdc4 pathway, which controls c-Myc. Cancer Research, 72(22), 6065–6075. https://doi.org/10.1158/0008-5472.CAN-12-2594

Lan, H., Tan, M., Zhang, Q., Yang, F., Wang, S., Li, H., Xiong, X., & Sun, Y. (2019). LSD1 destabilizes FBXW7 and abrogates FBXW7 functions independent of its demethylase activity. Proceedings of the National Academy of Sciences of the United States of America, 116(25), 12311–12320. https://doi.org/10.1073/pnas.1902012116

Li, L., Sarver, A. L., Khatri, R., Hajeri, P. B., Kamenev, I., French, A. J., Thibodeau, S. N., Steer, C. J., & Subramanian, S. (2014). Sequential expression of miR-182 and miR-503 cooperatively targets FBXW7, contributing to the malignant transformation of colon adenoma to adenocarcinoma. The Journal of Pathology, 234(4), 488–501. https://doi.org/10.1002/path.4407

Li, Y., Hu, K., Xiao, X., Wu, W., Yan, H., Chen, H., Chen, Z., & Yin, D. (2018). FBW7 suppresses cell proliferation and G2/M cell cycle transition via promoting γ-catenin K63-linked ubiquitylation. Biochemical and Biophysical Research Communications, 497(2), 473–479. https://doi.org/10.1016/j.bbrc.2018.01.192

Ma, L.-M., Liang, Z.-R., Zhou, K.-R., Zhou, H., & Qu, L.-H. (2016). 27-Hydroxycholesterol increases Myc protein stability via suppressing PP2A, SCP1 and FBW7 transcription in MCF-7 breast cancer cells. Biochemical and Biophysical Research Communications, 480(3), 328–333. https://doi.org/10.1016/j.bbrc.2016.10.038

Maddipati, R., Norgard, R. J., Baslan, T., Rathi, K. S., Zhang, A., Saeid, A., Higashihara, T., Wu, F., Kumar, A., Annamalai, V., Bhattacharya, S., Raman, P., Adkisson, C. A., Pitarresi, J. R., Wengyn, M. D., Yamazoe, T., Li, J., Balli, D., Lariviere, M. J., … Stanger, B. Z. (2022). MYC Levels Regulate Metastatic Heterogeneity in Pancreatic Adenocarcinoma. Cancer Discovery, 12(2), 542–561. https://doi.org/10.1158/2159-8290.CD-20-1826/673841/AM/MYC-LEVELS-REGULATE-METASTATIC-HETEROGENEITY-IN

Makohon-Moore, A. P., Zhang, M., Reiter, J. G., Bozic, I., Allen, B., Kundu, D., Chatterjee, K., Wong, F., Jiao, Y., Kohutek, Z. A., Hong, J., McMahon, B., Wood, L. D., Hruban, R. H., Nowak, M. A., Papadopoulos, N., Kinzler, K. W., Vogelstein, B., & Iacobuzio-Donahue, C. A. (2016). Limited heterogeneity of known driver genes among the metastases of individual pancreatic cancer patients. (Submitted), 49(3), 358–366. https://doi.org/10.1038/ng.3764.Limited

Mansour, M. R., Sanda, T., Lawton, L. N., Li, X., Kreslavsky, T., Novina, C. D., Brand, M., Gutierrez, A., Kelliher, M. A., Jamieson, C. H. M., von Boehmer, H., Young, R. A., & Look, A. T. (2013). The TAL1 complex targets the FBXW7 tumor suppressor

by activating miR-223 in human T cell acute lymphoblastic leukemia. The Journal of Experimental Medicine, 210(8), 1545–1557. https://doi.org/10.1084/jem.20122516

Roe, J., Hwang, C., Somerville, T. D. D., Park, Y., Tuveson, D. A., Vakoc, C. R., Young, C. M., Miyabayashi, K., Filippini, D., Creighton, B., Maiorino, L., Burkhart, R. A., Buscaglia, J. M., Kim, E. J., Grem, J. L., Lazenby, A. J., Grunkemeyer, J. A., Hollingsworth, M. A., Grandgenett, P. M., … Park, Y. (2017). Enhancer Reprogramming Promotes. 875–888. https://doi.org/10.1016/j.cell.2017.07.007

Sanborn, J. Z., Chung, J., Purdom, E., Wang, N. J., Kakavand, H., Wilmott, J. S., Butler, T., Thompson, J. F., Mann, G. J., Haydu, L. E., Saw, R. P. M., Busam, K. J., Lo, R. S., Collisson, E. A., Hur, J. S., Spellman, P. T., Cleaver, J. E., Gray, J. W., Huh, N., … Cho, R. J. (2015). Phylogenetic analyses of melanoma reveal complex patterns of metastatic dissemination. Proceedings of the National Academy of Sciences of the United States of America, 112(35), 10995–11000. https://doi.org/10.1073/pnas.1508074112

Song, Y., Lai, L., Chong, Z., He, J., Zhang, Y., Xue, Y., Xie, Y., Chen, S., Dong, P., Chen, L., Chen, Z., Dai, F., Wan, X., Xiao, P., Cao, X., Liu, Y., & Wang, Q. (2017). E3 ligase FBXW7 is critical for RIG-I stabilization during antiviral responses. Nature Communications, 8(1), Article 1. https://doi.org/10.1038/ncomms14654

Spruck, C. H., Strohmaier, H., Sangfelt, O., Müller, H. M., Hubalek, M., Müller-Holzner, E., Marth, C., Widschwendter, M., & Reed, S. I. (2002). HCDC4 gene mutations in endometrial cancer. Cancer Research, 62(16), 4535–4539.

Tajik, M., Baharfar, M., & Donald, W. A. (2022). Single-cell mass spectrometry. Trends in Biotechnology, 0(0). https://doi.org/10.1016/j.tibtech.2022.04.004

Teplyuk, N. M., Uhlmann, E. J., Wong, A. H.-K., Karmali, P., Basu, M., Gabriely, G., Jain, A., Wang, Y., Chiocca, E. A., Stephens, R., Marcusson, E., Yi, M., & Krichevsky, A. M. (2015). MicroRNA-10b inhibition reduces E2F1-mediated transcription and miR-15/16 activity in glioblastoma. Oncotarget, 6(6), 3770–3783. https://doi.org/10.18632/oncotarget.3009

Uhlén, M., Björling, E., Agaton, C., Szigyarto, C. A.-K., Amini, B., Andersen, E., Andersson, A.-C., Angelidou, P., Asplund, A., Asplund, C., Berglund, L., Bergström, K., Brumer, H., Cerjan, D., Ekström, M., Elobeid, A., Eriksson, C., Fagerberg, L., Falk, R., … Pontén, F. (2005). A human protein atlas for normal and cancer tissues based on antibody proteomics. Molecular & Cellular Proteomics: MCP, 4(12), 1920–1932. https://doi.org/10.1074/mcp.M500279-MCP200

Uhlén, M., Fagerberg, L., Hallström, B. M., Lindskog, C., Oksvold, P., Mardinoglu, A., Sivertsson, Å., Kampf, C., Sjöstedt, E., Asplund, A., Olsson, I., Edlund, K., Lundberg, E., Navani, S., Szigyarto, C. A.-K., Odeberg, J., Djureinovic, D., Takanen, J. O., Hober, S., … Pontén, F. (2015). Proteomics. Tissue-based map of the human proteome. Science (New York, N.Y.), 347(6220), 1260419. https://doi.org/10.1126/science.1260419

Whittle, M. C., Izeradjene, K., Geetha Rani, P., Feng, L., Carlson, M. A., DelGiorno, K. E., Wood, L. D., Goggins, M., Hruban, R. H., Chang, A. E., Calses, P., Thorsen, S. M., & Hingorani, S. R. (2015). RUNX3 controls a metastatic switch in pancreatic ductal adenocarcinoma. Cell, 161(6), 1345–1360. https://doi.org/10.1016/j.cell.2015.04.048

Yachida, S., Jones, S., Bozic, I., Antal, T., Leary, R., Fu, B., Kamiyama, M., Hruban, R. H., Eshleman, J. R., Nowak, M. A., Velculescu, V. E., Kinzler, K. W., Vogelstein, B., & Iacobuzio-Donahue, C. A. (2010). Distant metastasis occurs late during the genetic evolution of pancreatic cancer. Nature, 467(7319), 1114–1117. https://doi.org/10.1038/nature09515

Yeh, C. H., Bellon, M., & Nicot, C. (2018). FBXW7: A critical tumor suppressor of human cancers. Molecular Cancer, 17(1), 1–19. https://doi.org/10.1186/s12943-018-0857-2

Yokobori, T., Mimori, K., Iwatsuki, M., Ishii, H., Tanaka, F., Sato, T., Toh, H., Sudo, T., Iwaya, T., Tanaka, Y., Onoyama, I., Kuwano, H., Nakayama, K. I., & Mori, M. (2012). Copy number loss of FBXW7 is related to gene expression and poor prognosis in esophageal squamous cell carcinoma. International Journal of Oncology, 41(1), 253–259. https://doi.org/10.3892/ijo.2012.1436

Zhang, Q., Karnak, D., Tan, M., Lawrence, T. S., Morgan, M. A., & Sun, Y. (2016). FBXW7 Facilitates Nonhomologous End-Joining via K63-Linked Polyubiquitylation of XRCC4. Molecular Cell, 61(3), 419–433. https://doi.org/10.1016/j.molcel.2015.12.010