OVERVIEW. Familial cancers are rare (5-10%) but they represent a unique opportunity to study complex cellular events associated with tumor formation. To date, about ten hereditary kidney cancer syndromes have been identified and related to causative genes. Von Hippel-Lindau (VHL) syndrome is an autosomal dominant disease caused by germline mutations on the VHL gene resulting in benign and malignant lesions in a variety of organs such as eye, ear, brain, adrenal gland, pancreas and kidney[1-3]. The prevalence of VHL disease is between 1/36,000 and 1/50,000 births and affect similarly men and women of all ethnicities[4-6]. DNA testing and genetic counseling in family members facilitate the diagnosis. Genetic studies on cohorts of patients classify the disease into two subtypes based on the clinical manifestations. In addition, VHL databases from centralized centers worldwide are very helpful to understand the disease and facilitate potential benefits for patient and disease management. However, there is no universal treatment since clinical manifestations are different in each patient. Active surveillance is a key element to receive best treatment. Research is needed to provide novel insights into VHL-associated tumorigenesis and improve therapeutic approaches.

RESEARCH JUSTIFICATION. Our goal is to demonstrate the potential of targeting lysosomes in hereditary kidney cancer or other malignant tumors associated with VHL disease. The rationale is based on our previous published studies on sporadic clear cell Renal Cell Carcinoma (ccRCC) where we demonstrated the possibility of selectively targeting VHL loss compared to ccRCC with the functional VHL gene[7-9]. Indeed, we identified STF-62247 that showed specific cytotoxicity for ccRCC with a loss of VHL compared to RCC with a functional gene. STF-62247 blocks late-stages of autophagy by causing massive enlargement of endocytic compartments in VHL-inactivated ccRCC along with a reduction in lysosome numbers, leading to cell death[10]. Conversely, VHL-functioning cells were able to surmount a late-stage block in autophagy suggesting a role for VHL in maintaining lysosome turnover. More recently, our unpublished data revealed the PIKfyve lipid kinase as STF-62247 target. PIKfyve activity is required to maintain the dynamic equilibrium of endocytic compartments and lysosomal functionality. PIKfyve is the only enzyme able to phosphorylate PI3P into $PI(3,5)P_2$, a low-abundance phosphoinositide present at the lysosomal membranes. $PI(3,5)P_2$ has been shown to regulate lysosome dynamics and size, fission/fusion events, calcium channels and lysosome reformation[11,12].

OBJECTIVES. Our **hypothesis** is that VHL mutations associated with VHL-disease can be specifically targeted through PIKfyve inhibition as novel therapeutic approach. Our **specific aims** are:

i) To generate *in vitro* models of germline VHL-mutations and evaluate the potential of STF-62247 to target VHL disease;
ii) To study autophagy and lysosome physiology in diverse subtypes of VHL disease;
iii) To examine autophagy and lysosomal protein expressions in tissues from patients with hRCC associated with VHL disease.

RESEARCH PROGRESS. To achieve aim 1, we generated cell models expressing 10 VHL mutations spanning the entire coding sequence and associated with each subtype of the disease. We chose among the most frequently mutated VHL positions (Fig.1A). These mutations were incorporated into 786.0 cells, a ccRCC cell line that do not express VHL (stop codon). We used western blot analyses to verify VHL expression. DNA sequencing confirmed the presence of the mutation. Expression of the hypoxia-inducible factor 2 (HIF-2α) was also performed to evaluate whether the VHL mutation as the ability to conserve its HIF regulatory function. Then, we tested efficacy of STF-62247 and PIKfyve inhibitors on cell survival in all generated models. YM201636, vacuolin-1, APY0201 and apilimod are small molecules with morpholino-azine group known as PIKfyve inhibitors[13-15]. Our results indicated that mutations associated with type 1 and type 2B VHL disease are more sensitive to STF-62247 and PIKfyve

inhibitors (Fig.1B). When studying cells expressing VHL type 2A disease, our results indicate that cells harboring Tyr98His mutation are significantly less sensitive to these inhibitors (Fig.1B). Similar results were obtained Leu188Val mutant cells (Type 2C VHL disease) (Fig.1B). These results are corroborated using YM201636 and APY0201 (data not shown). These experiments are still ongoing but suggest that specific VHL point mutation associated with low risk of ccRCC are less sensitive to our approach. It is not possible at this moment to associated VHL disease type with STF-62247 sensitivity. Mutation location, interaction with protein partners could explain the difference in cytotoxicity of PIKfyve inhibitors and could be investigated through in silico analysis. Combination of STF-62247 with approved drugs for VHL disease such as belzutifan, sunitinib and pazopanib will be tested this summer.

The goal of proposed studies in aim 2 investigate whether autophagy and lysosomal processes are affected by VHL-disease associated mutations. Lysosomes are degradative organelles that participate in intracellular signaling, nutrient sensing and cellular metabolism[16,17]. Lysosomes respond to stimuli by adjusting their size, number, and position, which impact their activity[18-20]. Perinuclear localization of lysosomes increases their activities and promotes fusion with autophagosomes. Oppositely, lysosomes closer to the plasma membrane assure proximity to signaling receptors in presence of nutrients[19,21]. Compromised lysosomal positioning and activity are observed in various diseases including cancer[22,23]. Using western blot analyses, our results showed that LC3B protein expression, a marker of autophagy, and Lamp1, which is a lysosomal membrane protein, are differently expressed in mutated cells while PIKfyve expression is more stable (Fig. 2A,B). As observed in sporadic ccRCC, LC3B increased in response to STF-62247 and PIKfyve inhibitors while PIKfyve total expression is less influenced. Efficacy of STF and PIKfyve inhibitors was demonstrated by the reduced level of $PI(3,5)P_2$ (data not shown). Intriguingly, PIKfyve band is higher in STF-treated suggesting a post-trancriptional modification, which is under investigation but could be related to its activity (Fig. 2C). Finally, lysosomes were stained with Lamp1 antibody and lysosome numbers and distance from the nucleus were quantified using Fiji (Fig. 3). Results from control cells without indicated that cells with VHL mutation that are sensitive to STF-62247 have lower number of lysosomes. Furthermore, lysosomes in these cells are found in the perinuclear region. Oppositely, cells that are less sensitive to STF showed higher number of lysosomes which are more dispersed inside the cytoplasm. These results will be further investigated in response to PIKfyve inhibitors and in all generated mutants.

Until now, we were able to generate 10 cell models stably expressing VHL-disease mutations which have been used to assess cell survival in response to STF-62247 and PIKfyve inhibitors, and to analyze lysosome physiology. Our findings are promising and indicated significant difference in survival response to PIKfyve inhibitors. Importantly, cells with VHL mutations presenting higher lysosome number survive to STF-62247 treatment. Surprisingly, our results suggest that STF-62247 induces post-translational modifications that could affect PIKfyve activity. We are grateful to the Canadian VHL-Alliance and the Cancer Research Society to fund this project.

STUDENT RECRUITMENT. Dominique Comeau and Nadia Bouhamdani were postdoc in the lab and generated cell models and participated in lysosomal studies. Moreover, Chloé Girouard, a Master's student in biochemistry at the Université de Moncton was recruited to study this research project. Another student will join Chloé this summer.

CONFERENCES AND PUBLICATION. Chloé presented this work at the annual New Brunswick Health Research Conference in November 2021 where she won the second prize (poster). She also participated at the «congrès des jeunes chercheurs de l'Université de Moncton», in an online event (March 2021). Moreover, Chloé will participate to the VHL alliance international conference in november in Toronto. We are planning to submit a publication later this year.

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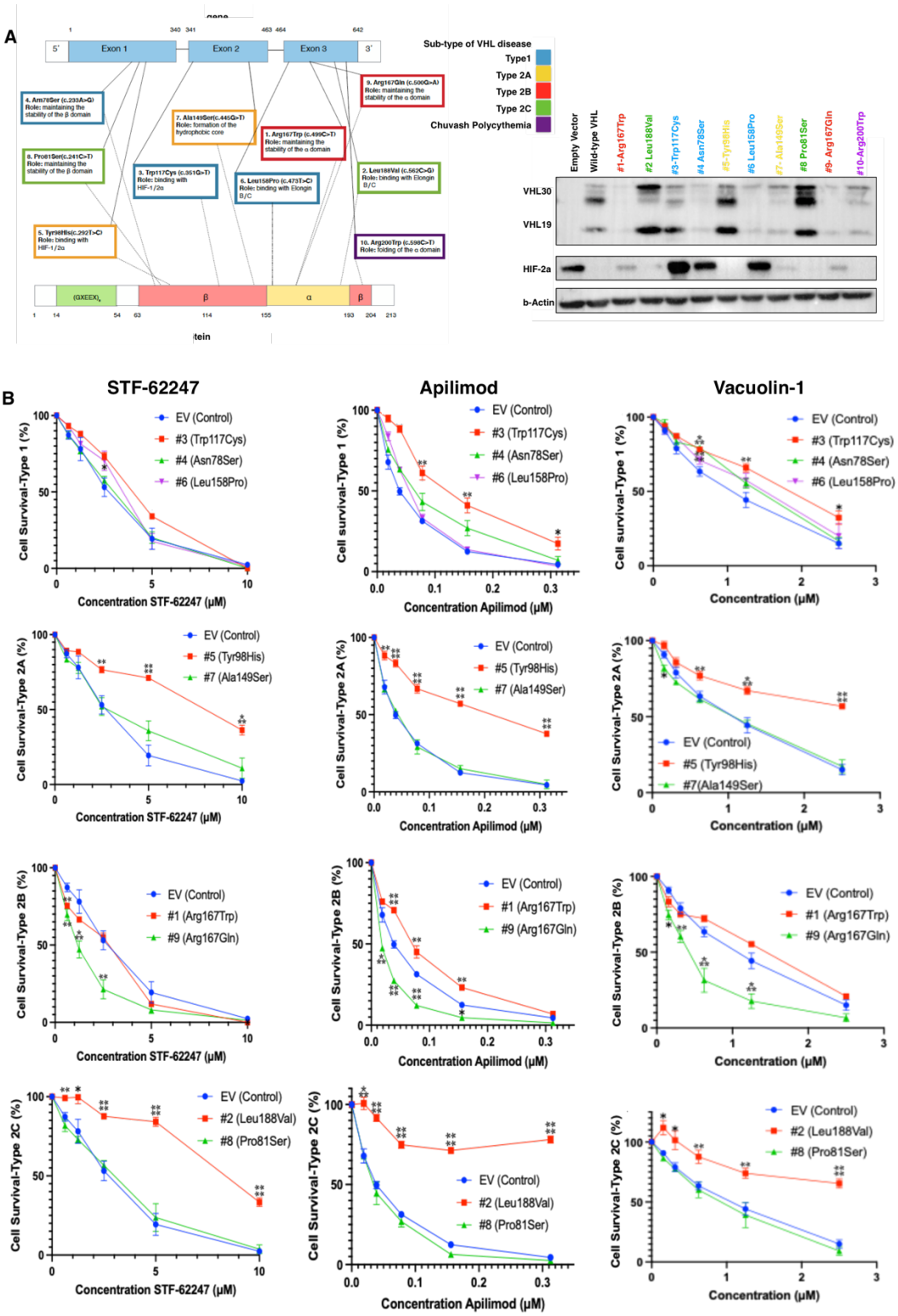

Figure 1. Sensitivity os STF-62247 and PIKfyve inhibitors in VHL mutants associated with VHL disease. A. left. Localization of VHL mutants and association with VHL function; right. Western blot analyses showing VHL and HIF-2a expression in 786.0 cells expressing VHL mutants. B. Clonogenic survival assays in response to STF-62247, apilimod and vacuolin-1 performed in models generated in A. Survival assays were performed in rechnical and biological triplicate. Error bars are SEM.

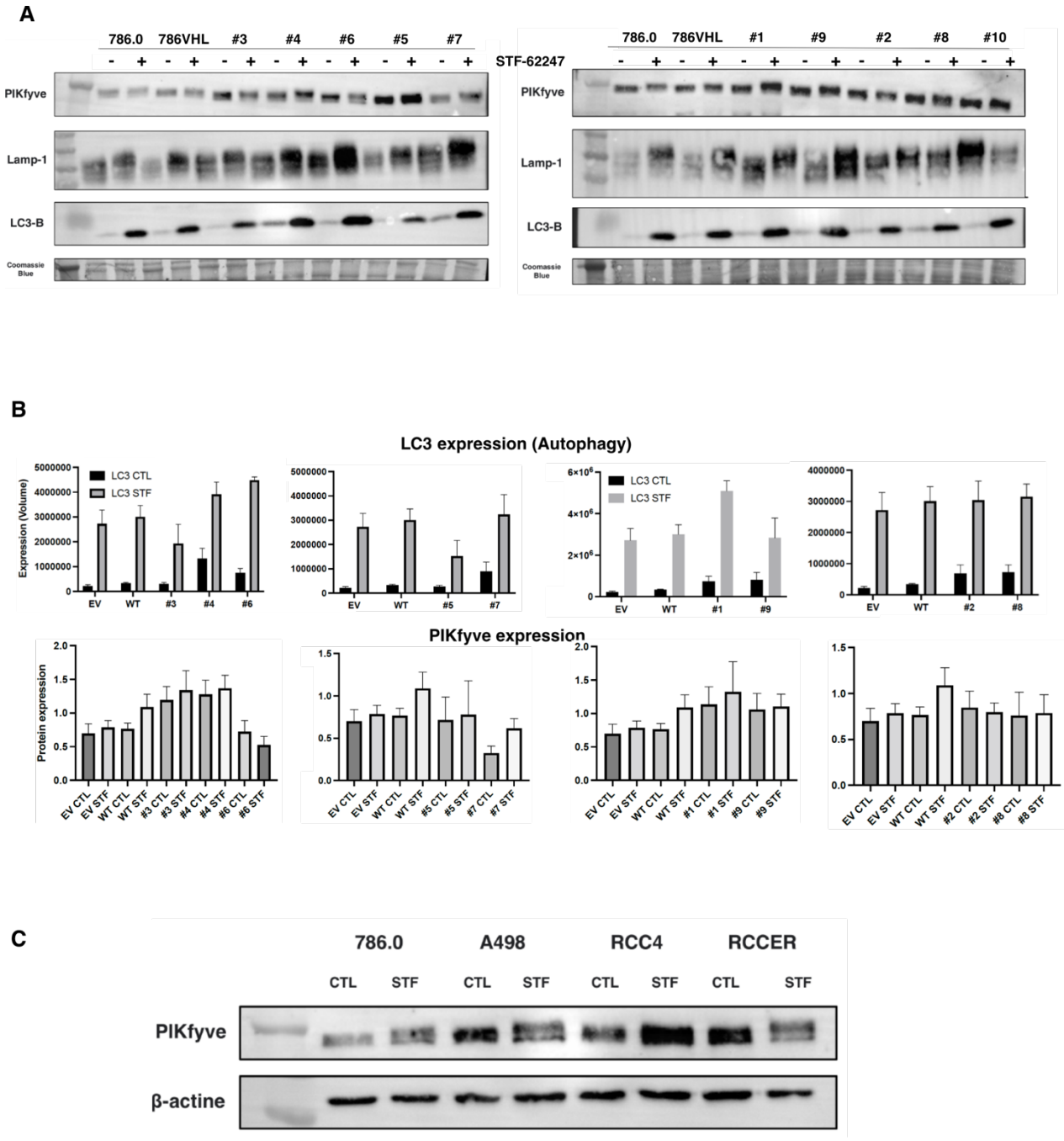

Figure 2. Autophagy and Lysosomal protein expression in cells expressing VHL germline mutation. A. Western blot analyses in cell models associated with VHL disease in response to STF-62247. Cells wre treated at 2.5 μM of STF-62247 for 48 hrs. B. Quantification of LC3B and PIKfyve expression showed in A. C. PIkfyve expression in ccRCC models in response to STF-62247. Each experiment was performed in biological triplicate. Error bars are SEM.

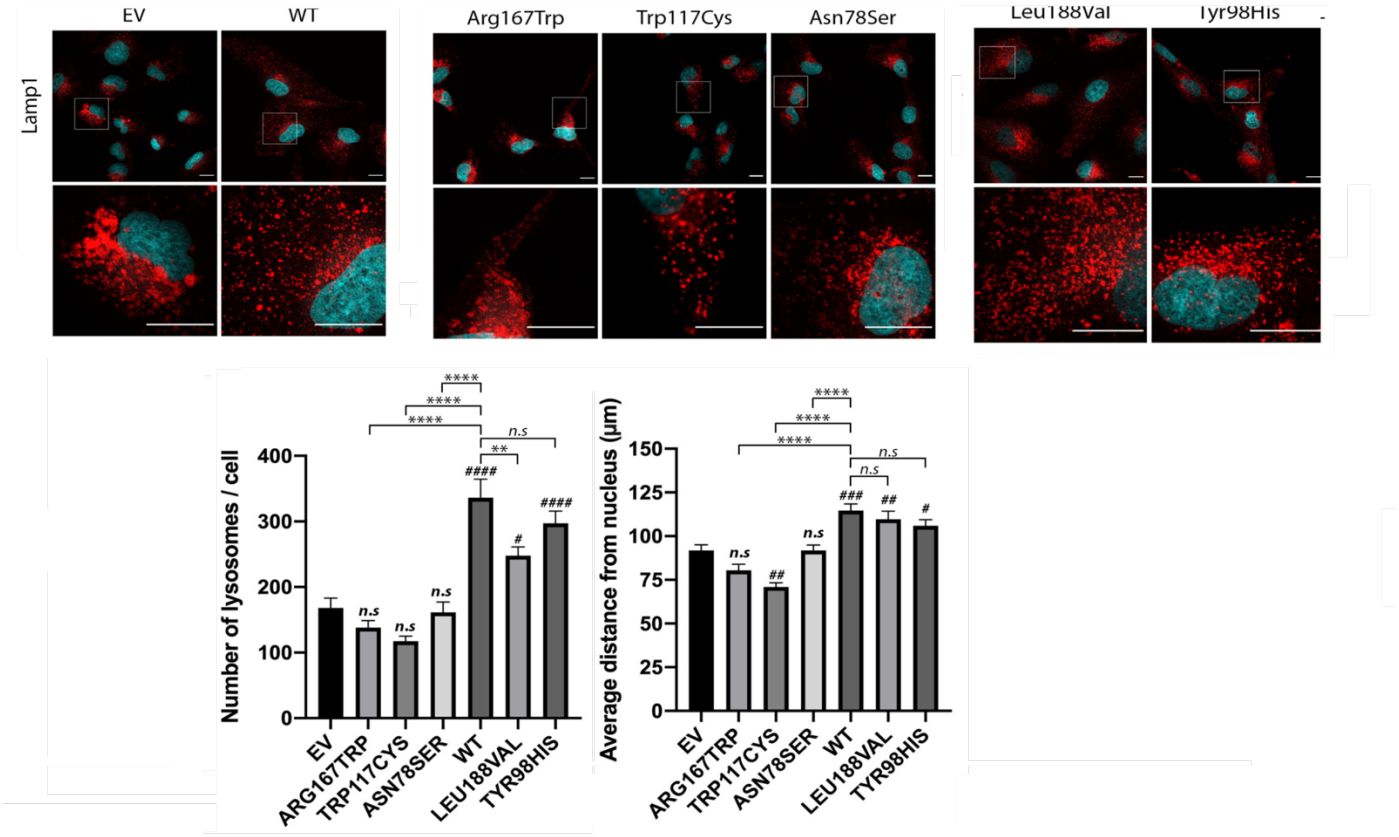

Figure 3. Lysosome number is lower is cells sensitive to STF-62247. Control VHL-mutated cells were stained with Lamp-1 (lysosomes) and DAPI (nucleus) and visualized by confocal microscopy. Lysosome number and localization were quantified using Fiji software. Each experiment was performed in biological triplicate. Quantification was obtained from at least 3 images per condition. Error bars are SEM.