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Live Imaging of Cysteine-Cathepsin Activity Reveals Dynamics of Focal Inflammation, Angiogenesis, and Polyp Growth

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Abstract

It has been estimated that up to 30% of detectable polyps in patients regress spontaneously. One major challenge in the evaluation of effective therapy of cancer is the readout for tumor regression and favorable biological response to therapy. Inducible near infra-red (NIR) fluorescent probes were utilized to visualize intestinal polyps of mice hemizygous for a novel truncation of the Adenomatous Polyposis coli (APC) gene. Laser Scanning Confocal Microscopy in live mice allowed visualization of cathepsin activity in richly vascularized benign dysplastic lesions. Using biotinylated suicide inhibitors we quantified increased activities of the Cathepsin B & Z in the polyps. More than ½ of the probe signal was localized in CD11b+Gr1+ myeloid derived suppressor cells (MDSC) and CD11b+F4/80+ macrophages infiltrating the lesions. Polyposis was attenuated through genetic ablation of cathepsin B, and suppressed by neutralization of TNFα in mice. In both cases, diminished probe signal was accounted for by loss of MDSC. Thus, in vivo NIR imaging of focal cathepsin activity reveals inflammatory reactions etiologically linked with cancer progression and is a suitable approach for monitoring response to therapy.

Introduction

Colonic and intestinal adenocarcinomas arise as a direct result of the loss of function of the adenomatous polyposis coli (APC) gene and stabilization of β-catenin. Rodent models of hereditary colon cancer faithfully reproduce the histopathology of familial adenomatous polyposis coli (FAP) [1,2] and provide opportunities for investigating secondary events that modulate genetic predisposition to colon cancer [3]. Accumulating evidence suggests that inflammation has causative roles in carcinogenesis [4]. While chronic inflammation can predispose to DNA damage and carcinogenesis, there is evidence to suggest that inflammation is a necessary component of tumor growth. In line with this notion, treatment of APC defective Min mice [5] with cyclooxygenase-2 (COX2) inhibitors results in a transient suppression of polyposis [6,7], an observation that parallels the response of colon cancer patients to similar treatments [8]. Furthermore, anti-TNFα, or the transfer of CD4+CD25+CD45RBlow regulatory T (Treg) cells, both hinder polyp growth in mice [9]. Together these observations strongly argue in favor of a causative link between inflammatory reactions and genetically induced colon cancer, opening possibilities for monitoring and targeting cancer associated inflammation for diagnostic and therapeutic purposes.

Proteolytic enzymes play essential roles in tumor growth, angiogenesis, and invasion. Cathepsins of the cysteine protease family and in particular cathepsin-B are commonly active in the tumor microenvironment, contributing to the regulation of angiogenesis and invasion during cancer progression [10,11]. We have shown that optical in vivo imaging of cathepsin B activity using near infra-red mechanism-based probe allows for highly sensitive detection of adenomatous polyps in mice with direct reflectance imaging [12,13]. The cathepsin inducible fluorescent probe (ProSense 680) is a composite polymer containing a poly-L-lysine backbone; on which quenched NIR (excitation 675 nm, emission 694 nm) fluorophore and several polyethyleno-glycol side-chains are attached. ProSense 680 is preferentially hydrolyzed by cathepsin B, but it can be activated through proteolysis by other cathepsins and other related proteases [14]. Using this approach, mouse polyps were specifically revealed by reflectance imaging [13,14,15] and by in vivo fluorescence endoscopy [16]. However, the cellular source of signal and biological meaning of the protease activity has remained enigmatic.

Here we used targeted in vivo analysis of cysteine cathepsins with ProSense 680. To investigate how specifically the probe activity demarcates areas of dysplasia and the relevance of probe signal to biological activity within the tumor we used the prototype
Olympus IV 100 scanning LASER intravital microscope to image intestinal lesions in a new mouse model of hereditary polyposis, APC\(^{\Delta 468}\) mice. We report that probe activation reflects the local density of pro-inflammatory cells infiltrating the lesion and amount of associated active enzyme at the tumor site. Furthermore, using cathepsin B deficient APC\(^{\Delta 468}\) (Ctsb\(^{-/-}\) APC\(^{\Delta 468}\)) mice, as well as, anti-inflammatory treatments, we provide evidence for a causative link between protease activity, inflammation and polyph growth. Altogether, the present study shows that NIR imaging of pre-neoplastic lesions using near infra red mechanism-based probes is a viable approach to detect biological activities etiologically connected with progressive tumor growth, and provides opportunities for monitoring biological response to effective therapy.

**Results**

**Mouse APC\(^{\Delta 468}\) Model: Morphology and Histopathology of Adenomatous Polyps**

A novel model of hereditary polyposis was generated by targeted deletion of exons 11 and 12 of the adenomatous polyposis coli (APC), causing truncation of the gene product at codon 468. The resulting mice (APC\(^{\Delta 468}\)) were backcrossed to C57BL/6j for at least 12 generations. Adenomatous polyps were found in abundance in the small intestine of the hemizygous APC\(^{\Delta 468}\) mice as early as 5 weeks of age, most frequently in the distal ileum (Fig. S1a&b). Colonic polyps were less frequent and increased with age. Morphologically, the polyps were undifferentiated (Fig. S2a&b), polyclonal (Fig. S2c), tubovillar (Fig. S2d&e), and were composed of tall hyperchromatic disorderly cells with cigar-shaped nuclei (Fig. S2f).

Closer histological examination revealed a rich infiltrate of hematopoietic cells, including granulocytes, mast cells, plasma cells, and lymphocytes (Fig. 1a–d). Two distinct populations of myeloid cells were significantly increased in the lesions: CD11b\(^+\)Gr1\(^+\) (MDSC), and CD11b\(^+\)F4/80\(^+\) macrophages (Fig. 1&2c). The MDSC infiltrate increased with age and tumor load (Fig. 2Aa compare with 2Ab; 2Ba&b), while macrophage density was elevated abruptly and remained high (Fig. 2Ac compare with 2Ad; Bc&d). In contrast, the intestine of ageing age mice showed little infiltration by MDSC, and had delayed increase in macrophages (Fig. 2B, black bars). As the APC\(^{\Delta 468}\) mice aged, anaemia and systemic inflammation developed contributing to a notable increase in the size of the spleen (Fig. S3a), which was in part accounted for by infiltrating MDSC and macrophages (Fig. S3b&c).

**Near infra red mechanism-based probes accurately mark areas of dysplasia**

Aged mice showing early signs of caehxia were injected with the ProSense 680 (2 nmoles/mouse) 24 hours before the imaging session. To image angiogenesis we employed the constitutively emitting AngioSense 750 probe and injected the mice just before the imaging session. Inside the tumor, the vessels expanded into large dead-end tumor vasculature (Fig. 3d) [17,18]. Tumor stroma was strongly stained; the vessels expanded into large dead-end areas of high signal intensity demarcating areas of dysplasia (Fig. 3b), and rich arrays of micro-vessels (Fig. 3c). The Image J software.

The increased signal of the ProSense 680 in microadenoma is due to more than 100 sections depth. The collected composite files of the z-stacks were analyzed with the Image J software.

In healthy bowel, the ProSense 680 image revealed a canonical honeycomb-like distribution of the signal depicting the axial image of the intestinal villi (Fig. 3a). The AngioSense 750 (Fig. 3c) and auto-fluorescence at 505–510 nm revealed similar honeycomb distributions, tracing the blood vessels and stroma (Fig. 3e). The image of micro-adenomas was drastically different, with broad areas of high signal intensity demarcating areas of dysplasia (Fig. 3b), and rich arrays of micro-vessels supplying the lesions.
To investigate whether the increased signal was due to more activity per cell or a greater number of cells with the same individual activity, we analyzed the volume and total intensity of the ProSense-680 particles. Their mean volume in the polyp z-stack (12900±40900  microm3, n = 92) was over 28 times that in the healthy intestine (4398±79.5  microm3, n = 91, Fig. 3g). The increase in total intensity of the ProSense-680 particles was directly proportional to the increase in volume of signal [Fig. 3i; 1/slope = 0.004540 for the APC WT polyps r²=1, total number of values 51, and 1/slope = 0.004539 for the healthy intestine r² = 0.9999, total number of values 60], suggesting that the increase in signal intensity was due to an increase in the numbers of cathepsin active cells. In agreement with this conclusion, we found no significant difference in the “specific intensity” (intensity per microm3) of the particles found in APC WT mice (242.9±0.01 units/microm3), and in WT mice (242.2±0.41 units/microm3), the P value between the sets being 0.1233 in the unpaired t test with Welch’s correction (the values of the size of the particles and their intensity were collected from the original 16-bit image). Furthermore, it was obvious that the centers of the ProSense-680 particles were distributed throughout the acquired slices of z-stacks, and were uniformly larger in volume and therefore brighter by about 2 orders of magnitude in the polyps (1.321*1010±8.873*109) as compared to the healthy surrounding tissue (8392±1257) (Fig. 3j; Suppl Video S1). Similar analysis of AngioSense-750 showed that the mean volume of the vessels in polyps was nearly 2.5 times higher (11270±9207  microm3, n = 101) than the mean volume of the vessels in the normal intestine tissue (4538±798  microm3, n = 81, Fig. 3h). Thus, increased vessel volume provides an independent means of visualizing early dysplasia, in accordance with published literature that links cathepsin activity with neovascularization [11,19,20].

ProSense 680 is activated by MDSC and macrophages

To reveal the cellular source of cathepsin activity, APC WT mice were stained in vivo by injecting i.v ProSense-680 24 hours prior to being sacrificed. The entire gut was then excised, washed, fixed and embedded in OCT and frozen for histology and immunofluorescence analyses. Immunofluorescence staining revealed overlap of both CD11b (AlexaFluor 488) and Gr1 (AlexaFluor 594) with ProSense-680 signal (Fig. 4a–e), in cells that were dispersed throughout the polyp stroma. The Image J “colocalization finder” in conjunction with the “nuclear counter” plug-in was utilized to analyze the fluorescent images. Figure 4d shows the result of the co-localization between the ProSense-680 (red) and CD11b (green) fluorescent images. Co-localized pixels were revealed as white spots, and correspond to the 22/42 CD11b+ cells and 22/36 ProSense-680+ cells. Figure 4e is the outcome of the colocalization analysis between ProSense-680 (red) and the Gr1 (green) fluorescent images. Again, co-localized pixels were revealed as white spots, and this time correspond to the 11/45 Gr1+ cells and 11/36 ProSense-680+ cells.

To further characterize cathepsin probe-active cells, total Mono-Nuclear Cells (MNCs) were prepared from intestine of 5-month-old APC WT mice. These were incubated ex vivo with 0.2 nmoles/ml ProSense-680 for one hour and after cell surface staining subjected to FACS analysis. MDSC and macrophages accounted for over 75% of the ProSense+ MNCs (Fig. 4f&g). Altogether, 11 ±0.69%, of the living cells (mice n = 6) were stained with the cathepsin activated probe, of which 3.4±2.06 were MDSC and 5.0±3.04% macrophages (Fig. 4i). For control, the MNCs were incubated with 50 μg/ml JPM-565 that is a general cathepsin inhibitor in RPMI 1640 for an hour at 37°C in the presence of 5% CO2 and then stained with 0.2 nmoles/ml ProSense 680. This resulted in a marked reduction in the intensity.
of staining of CD11b⁺ cells by Prosense 680 (Fig. S6a). In a typical experiment the frequency of Prosense 680+ cells was reduced from 3.49% to 1.59% (Fig. S6b). This result indicates that cathepsin activity was responsible for the ex vivo staining of myeloid cells by Prosense 680.

Cathepsin activity reports focal inflammatory reactions in dysplasia

To relate the signal to cathepsin activity, we quantified the amounts of active cysteine proteases of the cathepsin family in the polyps as compared to healthy surrounding tissue, and control healthy intestines. To quantify specific protease activity, we used DCG-04, a biotinylated derivative of the non-specific cathepsin inhibitor JPM-565 that interacts with the active site of cysteine cathepsins [21,22]. Tissue extracts from micro-dissected polyps and from healthy intestine tissues were incubated with DCG-04, and individual cysteine cathepsins were then identified by their relative molecular weights, after separating the extracted proteins by SDS-PAGE. This inhibitor has been used previously to measure active cathepsins B, S, L and Z (also known as cathepsin X) in cell extracts [21,22]. We used extracts from Cathepsin B deficient mice [23,24] as control. Comparing values from 6 mice per group confirmed significant (P<0.0001) up-regulation of active cathepsin B in polyps (17590±833 OD units) compared with neighboring tissue (7798±993 OD units), or from intestines of healthy age-matched mice (6879±651 OD units)(one tailed t test with Welch correction) (Fig. 5a). Treatment with anti-TNFα caused a significant drop in the levels of polyp specific active cathepsin B (7047±194 OD units; P<0.0001).

Similarly, we detected elevated levels of cathepsin Z in polyps (9886±971 OD) as compared with adjacent tissue (362±752 OD units; P=0.0005) or with wt intestine tissue (2265±595 OD units; P=0.0005) (one tailed t test with Welch correction). In accordance with previous reports [25], levels of active cathepsin Z were elevated in in the intestine of Ctsb⁻/⁻ APCΔ468 mice (6364±629 OD units) as compared to wt intestine (2266±595 OD units) (P=0.0179, t test with Welch correction). We were unable to detect cathepsin L and S. Cathepsin L has been reported to be unstable in extract [26].

Live Imaging of Cathepsin B activity Reveals Dynamics of Polyp Growth/Regression

These observations led us to conclude that the Prosense-680 signal was reporting cancer-associated inflammation. We had previously reported that treatment of APCΔ468 mice with anti-TNFα results in suppression of established polyps [27]. To assess
over 75% were either CD11b analyzed for CD11b staining. 

The "colocalization finder" plug-in produced images where the colocalized pixels appear white while the ProSense-680 was red (d&e), the CD11b was green (d, colocalization analysis of ProSense-680 and CD11b staining) and the Gr1 was green (e, colocalization analysis of ProSense-680 and Gr1 staining). x400 magnification. Arrows mark a CD11b/Gr1 ProSense 680+ cell. Representative FACS dot-plots of MNCs prepared from polyposis intestine and ex vivo stained with ProSense-680 followed by CD11b and Gr1 staining. The live MNCs were gated for ProSense-680+ cells, which were analyzed for CD11b/Gr1+ (f, MDSCs) and CD11b+/F4/80+ (g, macrophages) cells. Cumulative results of 6 FACS experiments showing % of CD11b/Gr1+ ProSense-680+ and CD11b+/F4/80+ProSense 680+ among total infiltrating MNCs. Note that among the ProSense-680+ cells (11±0.69% of total MNCs) over 75% were either CD11b/Gr1+ (3.4±0.6% of total MNCs) or CD11b+/F4/80+ (5.0±0.34% of total MNCs).

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Figure 4. The cellular source of cathepsin activity. Cryosections of ProSense-680 in vivo stained intestine from APC+/- mice were stained with antibodies to CD11b (AlexaFluor 488), Gr1 (AlexaFluor 594) and DAPI. The merged images of CD11b with DAPI (a, CD11b green, DAPI gray), Gr1 with DAPI (b, Gr1 red, DAPI gray), and ProSense-680 with DAPI (c, ProSense-680 blue, DAPI gray) were produced with the "RGB gray" plug-in of Image J. The "colocalization finder" plug-in produced images where the colocalized pixels appear white while the ProSense-680 was red (d&e), the CD11b was green (d, colocalization analysis of ProSense-680 and CD11b staining) and the Gr1 was green (e, colocalization analysis of ProSense-680 and Gr1 staining). 

The biological impact of cathepsin-B we compared genetic ablation of cathepsin B [23,24], with treatment of mice with anti-TNFα. Towards this end, we crossed APC+/- mice to cathepsin_B deficient mice. Western blot analysis with DCG-04 confirmed that polyps arising in cathepsin-B ablated mice were specifically devoid of cathepsin B activity (Fig. 5a,b), while cathepsin Z was not significantly altered. Interestingly, polyps of anti-TNFα treated mice also showed a selective decrease in cathepsin B, as compared to Z (Fig 5a,b).

Both ablation of cathepsin B and treatment of mice with anti-TNFα significantly reduced polyp density and size. Cathepsin B deficient mice had less polyps (39±2.1 adenoma of Ctsb+/− APC+/- mice n = 8, compared to 94±3.5 adenoma for APC+/- n = 16, P<0.0001 unpaired t test with Welsh correction), which had the trend to have smaller size (1.82±0.11 mm diameter for Ctsb+/− APC+/- mice n = 8, compared to 2.047±0.11 mm diameter for APC+/- mice n = 16, P=0.086) (Fig. 6a). Anti-TNFα treated mice also showed reduced numbers (34.3±4.49 adenoma, n = 8, P<0.0001, unpaired t test with Welsh correction) and sizes of polyps (1.516±0.022 mm in diameter, P<0.0001 unpaired t test with Welsh correction, n = 6) (Fig. 6c) as compared to untreated APC+/- mice.

FACS analysis revealed that intestines of Ctsb−/− APC+/- mice harbored 40% less ProSense-680+ MNCs (6.7±2.6%), as compared to the APC+/- (11%±2.69%, P=0.0037, Fig. 6b). This reduction can be attributed to a drop in the levels of MDSCs with ProSense-680 activity (mean frequency 0.56% as compared to 4.46%; P=0.0085, n = 6, unpaired t test with Welsh correction, Fig. 5c). The frequencies of ProSense-680+ CD11b+/F4/80+ cells remain unchanged (mean 5.36% to 5.03% respectively). MDSC density was also markedly reduced in anti-TNFα treated mice.

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Figure 5. Quantification of active cysteine cathepsins using a specific active site directed probe. Polyps from APC+/- Ctsb−/− APC+/-, and anti-TNFα treated APC+/- mice were micro-dissected, pooled, and extracts were incubated with DCG-04 prior to electrophoresis on a 4–12% gradient SDS gel and western blotting; healthy adjacent regions were similarly analyzed. Active Cathepsins were visualized with the use of chemiluminescence reagents. (a) A representative blot. (b) Average Optical Densities (OD) from each band were normalized with the OD of the β-actin protein, detected using a specific antibody. Open bars: Cathepsin Z, black bars: Cathepsin B.

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Figure 6. Polyps from APC+/- mice harbored 40% less ProSense-680+ MNCs (6.7±2.6%), as compared to the APC+/- (11%±2.69%, P=0.0037, Fig. 6b). This reduction can be attributed to a drop in the levels of MDSCs with ProSense-680 activity (mean frequency 0.56% as compared to 4.46%; P=0.0085, n = 6, unpaired t test with Welsh correction, Fig. 5c). The frequencies of ProSense-680+ CD11b+/F4/80+ cells remain unchanged (mean 5.36% to 5.03% respectively). MDSC density was also markedly reduced in anti-TNFα treated mice.
impacted the abundance of CD11b

triangles). Note that Cathepsin B deficiency predominantly

attenuate polyposis. To relate changes in ProSense-680

activity is an inherent component of cancer-associated inflamma-

tion in tumors in humans and experimental animals, making it an

attractive avenue for the imaging and possible treatment of cancer

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activities were focal and readily distinguished the dysplastic lesions

demarcation of dysplasia and the associated neo-angiogenesis.

Furthermore, we have demonstrated that this mode of imaging

dynamics of biological activity that is predictive of tumor growth

versus regression. In this study we have shown that cathepsin B

activity has been associated with a number of

reports cathepsin B activity, and reveals biological activity directly

and biological response to treatment. In this study we have shown that

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demarcation of dysplasia and the associated neo-angiogenesis.
using protease sensitive probes allows accurate detection of areas of dysplasia that may be undetectable by visible light imaging, due to size limits or anatomical features.

The source of cathepsin activity in tumors has been in the past debated, with some reports emphasizing that tumor cells are the major source of this activity. Here, we provide evidence based on histology and flow cytometry that clearly reveals tumor infiltrating myeloid lineage cells as the predominant source of this activity. This is a fortunate and useful finding, as cancer-associated inflammation is causatively linked with adenoma growth. Significantly, our analysis of fluorescent signals detected with in the lesions revealed that the local increase in signal intensity was due to increased numbers of cathepsin active cells rather than higher activity per cell. Furthermore, we could confidently and accurately detect this activity to a depth of at least 75 μm. Thus, the imaged signal was directly reporting the abundance of pro-inflammatory cells within the dysplasia.

We demonstrated that genetic ablation of cathepsin B results in suppression of tumor infiltrating pro-inflammatory cells, notable attenuation of polyposis, and a decrease in the fluorescent signal emanating from the lesions. Ablation of cathepsin B significantly increased the overall levels of active cathepsin Z. Previous reports suggest that cathepsin Z (also called cathepsin X) compensates for the levels of membrane bound cathepsin B, and is elevated in cathepsin B knock out mice [25]. Thus, attenuation of probe signal in polyps arising in cathepsin B knock mice suggests that the probe activity was relatively specific for cathepsin B. Furthermore, attenuation of polyposis in these mice suggests a specific requirement for cathepsin B in the progressive growth of polyps.

Surprisingly, cathepsin B deficiency predominantly affected the CD11b+Gr1+ MDSC infiltrate, and did not impact the CD11b+F4/80+ macrophage component of polyp infiltrating leukocytes. Both of these myeloid cell types contributed equally to the local activation of the ProSense 680 probe. Since preferential loss of MDSC correlated with polyph attenuation, we conclude that these cells were critically contributing to the progressive growth of dysplasia. Furthermore, we conclude that the decrease in cathepsin activated probe signal was largely due to the loss of MDSCs from areas of dysplasia.

The cytokine TNFα is regarded to be at the apex of inflammatory responses, promoting angiogenesis, mobilization of neutrophils and escalation of inflammation [28,29], including cancer associated inflammatory reactions [30]. Treatment of mice with anti-TNFα suppresses pathogen induced inflammatory bowel disease and inflammation triggered cancer [27,31,32]. We therefore postulated that if pro-inflammatory cells were the source of cathepsin activity and played a causative role in polyposis, then suppression of polyposis-associated inflammation should hinder progressive polyph growth, and this therapeutic effect should be reflected in a significant down-regulation of cathepsin activity. Treatment of mice with anti-TNFα resulted in a preferential loss of MDSC infiltrating the lesion, in increased apoptosis of the aberrant epithelial cells, and regression of the lesions. Accordingly, cathepsin-B activities as measured by western blot analysis and probe signal were significantly attenuated. Altogether, these observations establish live imaging of cathepsin B activity with sensitive near infrared probes as a highly specific method for detection of biological activity linked with progressive tumor growth.

Tumor associated neo-angiogenesis is considered to be a necessary pathological component of tumor growth and a viable target for therapeutic intervention. Pro-inflammatory cells are a recognized source of angiogenic factors, and suppression of inflammation is therefore expected to impact tumor associated inflammation. It is not known at what stage dysplasia triggers angiogenesis and how the dynamics of infiltrating leukocytes reflect the expansion or regression of blood vessels in the lesion. Here, taking advantage of a constitutively active near infrared probe we have imaged in a living animal the neo-vascularization of early dysplastic lesions by micro-vessels, and revealed the change in architecture of the vasculature entering the lesion. Using different fluorescent excitation and spectral separation of the images we succeeded in simultaneously imaging both angiogenesis and cathepsin B activity in the same tissue and 3-dimensional space. The visualization of the architecture of the lesion was further enhanced by concomitant imaging of auto-fluorescent signals that emanated largely from the tumor mucosa.

We were able to demonstrate that anti-inflammatory regiments such as ablation of cathepsin B or treatment of mice with anti-TNFα impacts the infiltration of dysplastic lesions by the microvasculature, and that loss of the vessels correlates with attenuation or regression of the lesions. Thus, near infrared imaging of blood microvasculature provides an independent means of monitoring progressive dysplasia, and response to effective therapeutic intervention.

In summary, we have shown that live imaging of mechanism-based near infrared probes allow simultaneous detection of independent biological activities that report progressive tumor growth. Our earlier studies have documented the feasibility of application of this mode of imaging to endoscopic monitoring of cancerous lesions. Here, we have provided mechanistic information on the source and biological significance of the signal. All together, we have documented a powerful advance in our visualization of tumor biology dynamics, which allows for sensitive monitoring cancer progression or favorable response to therapy.

**Materials and Methods**

**Microscope and the operational organization**

The prototype Olympus IV 100 LASER scanning intravital microscope was used to collect images from living tissues. This microscope has 4 lasers that with the use of a specialized array of filters can excite a wide variety of fluorochrome including the Cy5.5 (max excitation 680 nm, excited in this microscope with the 633 nm LASER, emission 680 nm) and Cy7 (excitation 749 nm, emission 790 nm). For this study the 4× and the 10× dry objectives were used. The emissions were separated in the desired spectrum through a set of mirrors and filters and collected from 3 Photomultipliers (PMT). Olympus FlowView software is used to compensate the channels and collect the FlowView images.

The microscope uses motors controlled by the software for the collection of the z-stacks used in this report. A 37°C table was available for the comfort of the anesthetized animals during operation.

**Live imaging probes**

To visualize in detail the cathepsin activity and its distribution along the intestine three probes were utilized, the ProSense 680 (ProSense 680), ProSense control 750 and a high molecular weight (250,000 g/molecule) probe linked with fluorochrome that is excited at 750 nm and emits at 790 nm (AngioSense750). ProSense 680 is a composite probe that is based on a poly-lysine backbone which on a number of fluorochrome molecules were linked on the side chain of the lysine moiety, as well as a number of MPEG molecules. The composite probe with the poly-L-lysine backbone intact does not emit fluorescence because of quenching, but when the backbone is hydrolyzed by cathepsins the fluorochrome emits a strong signal. ProSense control 750 has a poly-D-lysine backbone that cannot be hydrolysed by cathepsins.
and therefore remains quenched and is used as probe to visualize the non specific quenched accumulation of fluorescent dyes in areas of high vascularity. AngioSense 750 remains in the vessels during the normal experimentation period and delineates the vessels. The autofluorescent signal (505–510 nm) was used to visualize the outline of the tissue imaged.

**Mouse studies**

Cathepsin B knock-out mice have been reported before and were a kind gift [23,24]. Throughout the procedures in which animals were injected with the probe, as well as during the imaging session, mice were anaesthetized with inhalation of 1.5–2% mixture of isoflurane in oxygen.

Mice were retro-orbitally injected with the ProSense-680 24 hours before the experimental procedure (2 nm/mouse, 150 μl). Ten minutes before the imaging session the mice were injected with 100 μl of the AngioSense-750. According to our observations most of the probe remains in the vessels for at least 1 hour.

Mice were incised and a loop of the intestine was cut along the length and opened to allow imaging in z stacks from the inside out. The mice were constantly monitored for the rate of the respiration and the depth of their anesthesia. The typical imaging session has duration of 60–90 min, throughout the imaging session the mouse is alive and anaesthetized.

All animal experiments were approved by the Harvard Medical School Standing Committee on Animals (protocol 04064, Dr K. Khazaie), by the Dana Farber Cancer Institute IACUC (protocol 02033, Dr K. Khazaie) and the Northwestern University Animal Study Protocol 2007-1284 (Dr K. Khazaie) for “imaging proteolytic activity in colon cancer”.

**Imaging analysis software**

The collected z-stacks were in the form of multiple “.tiff” FlowView files and can be seen and split into channels with the USDA plug-in collection of ImageJ open source software. To analyze the particles we used the bundle of plug-in developed by the Bob and John Wright Cell Imaging Facility of the University of Ontario Canada (http://www.uhnresearch.ca/facilities/wcif/) and the related number assuming that the size distribution is Gaussian. The non-Regression Analysis to correlate the size of the polyps with their total activity in colon cancer”.

**Immunofluorescence analysis**

Mice were stained in vivo for 24 hours with 2 nm ProSense-680. The following day were sacrificed and the fillet-opened intestine was rolled and frozen in OCT. Cryosections were prepared (15 μm) and fixed in acetone (−20°C, 15 min), rehydrated in PBS and incubated with the primary antibodies (biotinylated anti-Cd11b, 1:75, and purified rat anti-Gr1, 1:75) antibodies for one hour. The slides were washed in PBS (3×3 min each) and incubated with the secondary antibodies (streptavidin AlexaFluor 594, and anti-rat IgG AlexaFluor 488, 1:75 each).

After an hour the sections were washed with PBS and mounted with antifade mounting solution that contains DAPI. A specific filter was utilized to visualize the Cy5.5 fluorochrome.

**Supporting Information**

**Figure S1** Distribution of adenomas in the APC<sup>468</sup> intestine. (a) the number of polyps plotted against their location throughout the length of the small intestine of 3 month-old mice. (b) Typical distribution of polyps along the length of the small intestine. (c) Number of polyps as a function of age of mice, plotted in three size groups. (d) Number of polyps as a function of age of mice. Found at: doi:10.1371/journal.pone.0002916.s001 (3.76 MB TIF)

**Figure S2** Histological properties of APC<sup>468</sup> polyps. (a) β-galactosidase staining Apc<sup>468</sup> adenoma. (b) PAS staining of a 6 μm paraffin section of an Apc<sup>468</sup> adenoma. The arrow indicates the characteristic purple staining of the mucus inside the goblet cells. (c) X-gal staining of T84 cell APC<sup>468</sup> R36R polyp; (d) H&E staining of a jellyroll preparation of an Apc<sup>468</sup> intestine. Magnification 50×. The arrow indicates a large adenoma. (e) 100× magnification of an area of the same section. The arrow indicates a small adenoma and the arrowhead a normal villus. (f) 400× magnification of the area surrounded with the white rectangular in (e). The arrow indicates an adenoma. Found at: doi:10.1371/journal.pone.0002916.s002 (3.95 MB TIF)

**Figure S3** Systemic inflammation in the APC<sup>468</sup> mice. (a) Increase in the weight of the spleen as the mice age (blue dots) as compared to the wt spleen weight (red dots) (P = 0.0003). The CD11b<sup>+</sup>Gr1<sup>+</sup> (b) and the CD11b<sup>+</sup>F4/80<sup>+</sup> cell numbers in the spleen of the Apc<sup>468</sup> (open bars) and the wt (black bars) mice. Found at: doi:10.1371/journal.pone.0002916.s003 (0.47 MB TIF)

**Figure S4** Autofluorescence intensity of the APC<sup>468</sup> adenoma. The distribution of the auto fluorescence alongside the z-stack. Apc<sup>468</sup> adenoma shown in Fig. 3f (black squares) as compared to the wt equivalent shown in Fig. 3e (open triangles). Found at: doi:10.1371/journal.pone.0002916.s004 (0.30 MB TIF)

**Figure S5** Cathepsin activity is required for unquenched ProSense 680 signal in the APC<sup>468</sup> adenoma. APC<sup>468</sup> mice were stained a day before the imaging session with 2 nmol/mouse of ProSense 680 and ProSense control 750. Stacks of 168 (1 μm/slice) slices were collected using the Olympus IV100, the UplanApo ×4 and ×2.5 electronic zoom using the channels for ProSense 680 and ProSense control 750. a. merge of the two channels of the 160th slice; b. the ProSense 680 image of the 160th slice; c. the ProSense control 750; d. the ratio of the ProSense 680 to the ProSense 750 signal. The mean intensity of the adenoma Region Of Interest (ROI, left rectangular) and the healthy surrounding ROI (right rectangular) was measured for the both channels in all 168 slices. The bar diagram shows that the this ratio is 49±1.4 for the adenoma ROI and 2.1±0.01 for the healthy. The sets of values are statistically significant (P<0.0001, t test with Welch correction). Found at: doi:10.1371/journal.pone.0002916.s005 (1.06 MB TIF)
Figure S6  The use of the JPM-565 inhibits the ex vivo staining of the MNCs with ProSense 680. Isolated APC\(^{465}\) MNCs were pretreated with 50 \(\mu\)g/ml of JPM-565 in RPMI 1640 for an hour in 37\(^\circ\)C and 5\% CO\(_2\). The positive control remained in the incubator treated with the carrier of the JPM-565. The cell suspension was then stained with 0.2 amoles ProSense 680. The stained cells were stained with PE anti CD11b antibody and DAPI to detect the dead cells. a. represents the flow cytometry of the ProSense 680\(^+\) CD11b\(^+\) cells untreated with JPM-565 and b. the treated equivalent.

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Video S1  Video of the z-stack of an APC\(^{465}\) adenoma. The stack consists of 72 RGB images obtained in three channels. Autothoerescence at 505–510 nm (blue), ProSense-680 at 694 nm (green), and vascularity contrast enhancing AngioSense-750 at 790 nm (red).

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Author Contributions
Conceived and designed the experiments: RW KK. Performed the experiments: EG CR RK KK. Analyzed the data: EG CHT TAB. Contributed reagents/materials/analysis tools: EG CHT RM JJ HLP RW. Wrote the paper: EG KK.

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