Materials and methods

Mice

Rosa26-rtTA, tetO-H2BGFP and AP2-CreER mice were generously provided by Rudolf Jaenisch, Elaine Fuchs and Daniel Metzger and Pierre Chambon, respectively. Rosa26-rtTA and tetO-H2BGFP mice were backcrossed to >95% C57BL/6 inbred background. OB/OB, R26R and Rosa26-lacZ mice were obtained from JAX. Mice were first maintained at a barrier facility in the Department of Molecular and Cellular Biology at Harvard University under animal protocol 93-15 and later maintained at a barrier facility at the Centre for Regenerative Medicine, Massachusetts General Hospital, Harvard University under animal protocols 2006N000104 and 2009N000050.

Genotyping

Genotyping was performed by adding a tail biopsy to 100µl DirectPCR (Viagen) with 30µg Proteinase K (Roche), incubating overnight at 55°C and denaturing Proteinase K for 20 minutes at 95°C. PCR primers specific to Rosa26-rtTA (A 5'-aaagtcgctctgagttgttat-3'; B 5'-gcgaagagtttgtcctcaacc-3'; C 5'-ggagcgggagaaatggatatg-3'), GFP (forward 5'-ctggtcgagctggacggcgacgtaaac-3'; reverse 5'-atgtgatcgcgcttctcgttgggg-3'), LacZ (forward, 5'-tttaacgccgtgcgctgttcg-3'; reverse, 5'-gatccagcgatacagcgcgtc-3') and AP2CreER (forward, 5'-cgaccaggttcgttcactca-3'; reverse, 5'-tgaccagagtcatccttagcg-3') amplified 300bp, 600bp, 600bp, 300bp and 400bp, fragments, respectively. PCR conditions: 95°C for 5 minutes, then 35 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 60 seconds, and finally 72°C for 5 minutes.

Doxycycline, BrdU and Tamoxifen

Doxycycline (Sigma) was added to drinking water at 1mg/ml and sweetened with sucrose (1%). Water bottles were changed weekly with freshly prepared solution. Animals were injected with 100 µl of 10mg/ml BrdU (Sigma) or 300µl BrdU Labeling Reagent (RPN201, Amersham). Tamoxifen (Sigma, http://www.sigmaaldrich.com) was resuspended in 90% corn oil, 10% ethanol at 50mg/ml, and heated to 55°C. Mice were injected with 3mg/40g mouse I.P.

Immunohistochemistry

Unless otherwise stated, wild-type mice were sacrificed at either 6- or 14-weeks of age. Abdominal fat tissue was dissected from mice, fixed in 4% paraformaldehyde/PBS solution for two hours at 4^0C and washed in PBS. Cryo samples were incubated in 30% sucrose/PBS solution overnight, embedded in OCT (Tissue-Tek) and stored at -80^0C. Paraffin samples were dehydrated through an ethanol series, washed three times in xylene, embedded in paraffin and stored at 4^0C. Frozen samples were sectioned at 30µm for staining, paraffin

samples were sectioned at 20μm. The following primary antibodies and dilutions were used: rabbit anti-Perilipin A/B (Sigma), 1:100; goat anti-Perilipin (Novus Biologicals), 1:100; rabbit anti-C/EBPα (Cell Signaling Technology), 1:50; goat anti-Adipsin (P-16) (Santa Cruz), 1:200; mouse IgG1 anti-Ki67 (BD PharMingen), 1:10; rabbit antiKi67 (Abcam), 1:500; mouse IgG2α anti-PCNA (Calbiochem, NA03), 1:1000; mouse IgG1 anti-BrdU antibody (clone B44) (BD Bioscience), 1:50; mouse IgG2α anti-BrdU (Amersham), 1:200; rat anti-CD31 (BD Bioscience), 1:100; PE-Cy5 mouse anti-Mac1 (CD11b) (eBioscience), 1:200; rabbit anti-Recoverin (Chemicon), 1:2,000., rabbit anti-Cre (Abcam) 1:200. Antigen retrieval was performed for BrdU staining using a Retriever2100 machine (Prestige Medical) in 10 mM citrate buffer (pH 6.0).

Secondary antibodies were Alexa donkey 488, 555, 594 and 647 anti-rabbit (Invitrogen), Alexa goat 488 and 555 anti-mouse IgG1 (Invitrogen), Alexa goat 488 and 555 anti-mouse IgG2α (Invitrogen), Alexa goat 488 and 555 anti-mouse (Invitrogen), Alexa goat 488, 555 and 594 anti-rat (Invitrogen), and Alexa donkey 488, 555, 568 and 594 anti-goat (Invitrogen), all were used at 1:500. To visualize nuclei, slides were stained with 0.5μg/ml DAPI (4',6-diamidino-2-phenylindole) and then mounted with either Aqua polymount (Polyscience, Inc) Mounting Medium or VectaShield Mounting Medium. Images were acquired using a Zeiss LSM510 Meta confocal microscope or a NIKON Eclipse TE2000-E. Ki67 and BrdU cell counts were conducted by epifluorescence microscopy or confocal microscopy.

Collagen embedding

Collagen embedding was performed on both floating adipocytes and diluted stromavascular cells (1:40). In both cases, cells were added to rat collagen type I hydrogel (42% collagen in DMEM), and then, following solidification, fixed in 4% paraformaldehyde for 15 minutes at room temperature and washed with PBS. Embedded adipocytes were immunostained and imaged with a Zeiss LSM510 Meta confocal microscope or a NIKON Eclipse TE2000-E T.

Embedded SV cells were stained for 16 hours with Histomark X-gal Substrate (KPL) at 37°C, washed with PBS, stained with DAPI and analyzed at a Leica DM6000B microscope. BODIPY labeling of collagen embedded fat cells was done by incubating the cells for 2 minutes with 1μM fluorescent fatty acid analog C1-BODIPY 500/510-C12 (1:2000 dilution in PBS) (Invitrogen) and then washing the cells with PBS.

For SV fractionation and culture, white abdominal adipose tissue was collected and minced into fine pieces (3 mm^2). To digest, adipose pieces were incubated in adipose isolation buffer (HBSS, 2% BSA, 12.5 mM HEPES) containing 1 mg/ml collagenase II and 0.2 mg/ml DNAseI at 37°C with constant shaking (~250rpm) for 1 hour. The filtered suspension through a 250mm nylon sieve was then centrifuged at 1000rpm for 5 minutes. The cell pellet (stromal-vascular fraction) was washed twice with 50 ml PBS and plated in a 6-well culture dish. The cells were cultured in Dulbecco's Modified Eagle Media (DMEM) with 10% FBS, 100units/ml penicillin, 100ug/ml streptomycin, and 50ug/ml gentamicin for 7 days. SV cultured cells were fixed in 2% formaldehyde, 0.2% glutaraldehyde, PBS for 20 minutes and then washed three times with PBS containing 2 mM MgCl2.

For lacZ staining, abdominal adipose tissue and *in vitro* cultured SV cells were fixed in gluteraldehyde, 5mM EGTA, 2mM MgCl2 in PBS for 30min and washed in PBS, (Slides were prepared by paraffin embedding.) All samples were incubated in X-gal staining buffer (1mg/ml X-gal, 5 mM $K_4Fe(CN)_6$-$3H_2O$, 5 mM $K_3Fe(CN)_6$, and 2 mM $MgCl_2$) at 37°C for 4 to 16 hours, post-fixed in formalin for 10 minutes and counterstained with DAPI if necessary. For cell counting, stained cells were photographed using a Leica DFC 420 camera (Leica Microsystems Ltd, United Kingdom) at 10x-20x magnification. The number of β-gal-positive cells and total nuclei were counted. Only the β-gal-positive cells with DAPI-stained nuclei were counted to exclude non-

specific staining. The percentage of β-gal-positive cells was expressed as a proportion to total nuclei.

FACS

For sorting of live, unfixed adipose cells, abdominal fat was dissected from mice, rinsed in PBS, and cut with scissors into small pieces of approximately 3 mm in diameter. The fat was then dissociated for 75 minutes in KRB solution (HBSS diluted 1x in PBS; 2% BSA, 12.5mM HEPES) with 1mg/ml Collagenase II (Sigma) and 0.2mg/ml DNAse I (Sigma) at 37ºC with high speed shaking. The homogeneous solution was filtered through a 250µM nylon sieve and centrifuged at 500xg at room temperature for 5 minutes. The upper layer was collected and washed with 50ml of KRB solution twice by spinning at 500xg for 20 seconds at room temperature. Cells were diluted in KRB 1:3 and sorted on a BD FACS Calibur (Becton Dickinson).

For sorting of fixed and stained adipose nuclei, abdominal fat was dissected from mice, rinsed in PBS, and cut with scissors into small pieces of approximately 3 mm in diameter. The fat was then dissociated for 75 minutes in KRB solution (HBSS diluted 1x in PBS; 2% BSA, 12.5mM HEPES) with 1mg/ml Collagenase II (Sigma) and 0.2mg/ml DNAse I (Sigma) at 37ºC with high speed shaking. The homogeneous solution was filtered through a 250µM nylon sieve and centrifuged at 500xg at room temperature for 5 minutes. The floating fraction is considered to consist primarily of buoyant adipocytes while the pelleted cells are termed stromal/vascular (SV) cells. Both fractions were collected separately and washed with 50ml of KRB solution twice by spinning at 500xg for 20 seconds at room temperature.

Nuclei were extracted by incubation 1:20 in cold lysis buffer (10mM TrisHCL buffer, 5mM MgCl2, 0.3 M sucrose and 0.4% NP-20) for 20 minutes on ice, pelleted at 2000xg and washed twice with lysis buffer. They were then layered

over a 1ml cushion of lysis buffer containing 0.88M sucrose and then collected by centrifugation at 5000xg for 10 minutes. Nuclei were fixed in 4% paraformaldehyde for 15 minutes, pelleted and washed in PBS by centrifugation at 6500xg for 1 minute and finally resuspended in a solution of 0.1% Tween 20 and 0.5% paraformaldehyde in PBS for one hour before being subjected to Ki67 (PE conjugated, BD PharMingen) and C/EBPα immunocytochemistry, stained with DAPI and sorted on a BD FACS LSRII (Becton Dickinson).

Results

NO LABEL-RETAINING CELLS ARE DETECTED IN ADIPOSE TISSUE

Tumbar *et al* engineered transgenic mice expressing H2BGFP from a tetracycline-responsive promoter (tetO-H2BGFP) to mark cells and assess their rates of division (Tumbar et al. 2004). To identify dividing cells within the fat cell population, we utilized this inducible H2BGFP strategy in combination with a constitutive promoter that expresses the reverse transactivator (rtTA) within adipose tissue. The Rosa26 locus is active in most cells of the mouse, suggesting that in the presence of doxycycline, Rosa26-rtTA should drive tetO-H2BGFP expression and label most mouse cells, including all the cell types found in adipose depots (Zambrowicz, B. P. et al. 1997). It has been previously shown that H2BGFP is diluted with cell division and distributed equally between daughter cells; that all cells, regardless of replicative activity, can be labeled by the inducible H2BGFP system; and that in post-mitotic retinal cell populations, H2BGFP is stable for at least six months (Brennand, K., et al. 2007) (Figure 1.3).

Administration of a doxycycline pulse to Rosa26-rtTA; tetO-H2BGFP mice resulted in labeling of diverse cell types, including but not limited to fat, intestine, liver, pancreas, bone marrow, muscle, skin, and retina (Brennand et al. 2007 and data not shown), but not cortical neurons, olfactory bulb, or spinal cord, possibly due to the inability of doxycycline to cross the blood–brain barrier (Hochedlinger et al., 2005). Under repressive conditions (without doxycycline), no expression was observed in these organs (Figures 1.5 and data obtained by Kristen Brennand, Melton lab, shown in Figures 1.3B)

The stability of H2BGFP can be most easily assessed in post-mitotic cells, where any loss of fluorescence with time can only be explained by degradation of the H2BGFP protein. Mammalian photoreceptor cells are post-mitotic and are not replaced over the lifespan of the animal; they are identified by their position within the outer nuclear layer of the retina and by expression of the calcium-binding protein recoverin (Dizhoor et al., 1991). Photoreceptor cells are labeled when Rosa26-rtTA; tetO-H2BGFP mice are subjected to a pulse of doxycyline (Figure 1.3A). Following a chase of 6 months (Figure 1.3A), H2BGFP can be detected in whole eyes and sectioned retinas with the same

imaging settings used to collect pulse data. Staining with recoverin verified that label retention is restricted to the photoreceptor layer of the retina (Figure 1.3A). Thus, the H2BGFP label is stable and retained in post-mitotic cells. Although, it is formally possible that the H2BGFP protein has a shorter half-life in fat cells than in post-mitotic photoreceptor cells.

This assay is designed to determine whether the cells within the adipose tissue are a heterogeneous population with regard to proliferative capacity. If that would be the case, cells exhibiting a high rate of proliferation will lose the H2BGFP label quickly as they replicate, while slowly dividing cells will retain the H2BGFP label. Alternately, if cells in the adipose tissue are a homogenous population, they would be expected to lose the H2BGFP label at similar rates (Figure 1.1). We observed uniform loss of the H2BGFP label with time, supporting the conclusion that all the cells in the adipose tissue contribute equally to cell growth and maintenance.

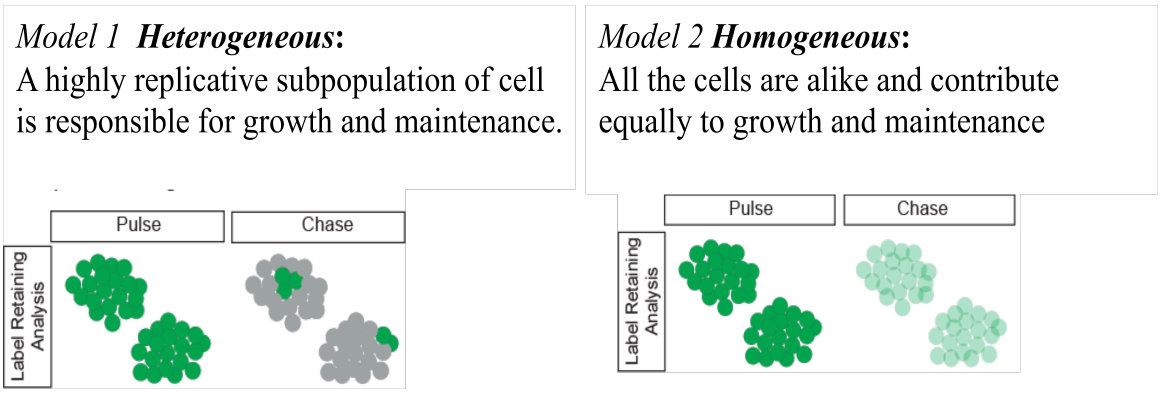

Figure 1.1 Two possible models for the growth and maintenance of adipose cells are predicted Pulse-chase analysis follows the loss of H2BGFP label (green) with fat cell division was used to study replication of cells within the adipose tissue. Model 1: The fat-cell population is heterogeneous, comprised of fast- and slow-dividing subpopulations. This model predicts that highly replicative cells will lose H2BGFP label quickly, while slowly dividing cells will fail to dilute H2BGFP label. Model 2: The fat cell population is homogeneous and all the cells divide at the same rate. This model predicts that all the cells in the adipose tissue will lose H2BGFP label uniformly.

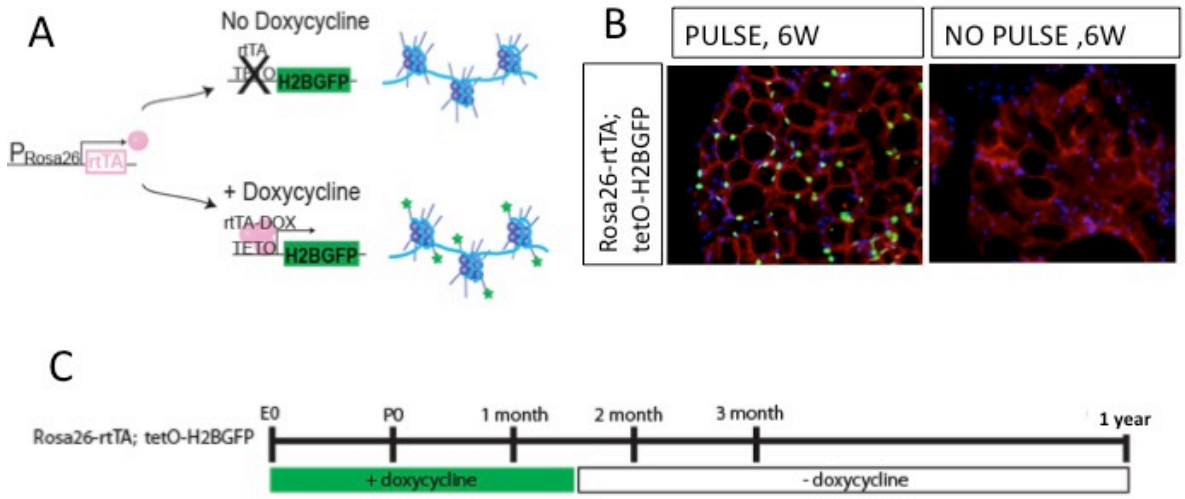

Figure 1.2. Experimental schematic for use of tetracycline-inducible Histone2B-GFP to identify label retaining cells in vivo. A. We have used the Tet-on system based on a reverse tetracycline-controlled transactivator, *rtTA*. *rtTA* is under the control of the promotor ROSA26 which is ubiquously expressed in the cells. rtTA protein is able to bind the TETO sequence only in the presence of Doxycycline effector so the transciption of the TRE-regulated H2BGFP transgene is expressed only in the presence of the doxycycline pulse. B. Doxycycline dependent expression of Rosa26-rtTA; tetO-H2BGFP expression in the adipose tissue. Perilipin, a marker of adipocytes, is shown in red. Up to 80% of cells are labeled following the pulse period, no adipose cells are labeled in the absence of pulse. Magnification 400x. C.C. Timeline for Rosa26-rtTA; tetO-H2BGFP pulse-chase experiments

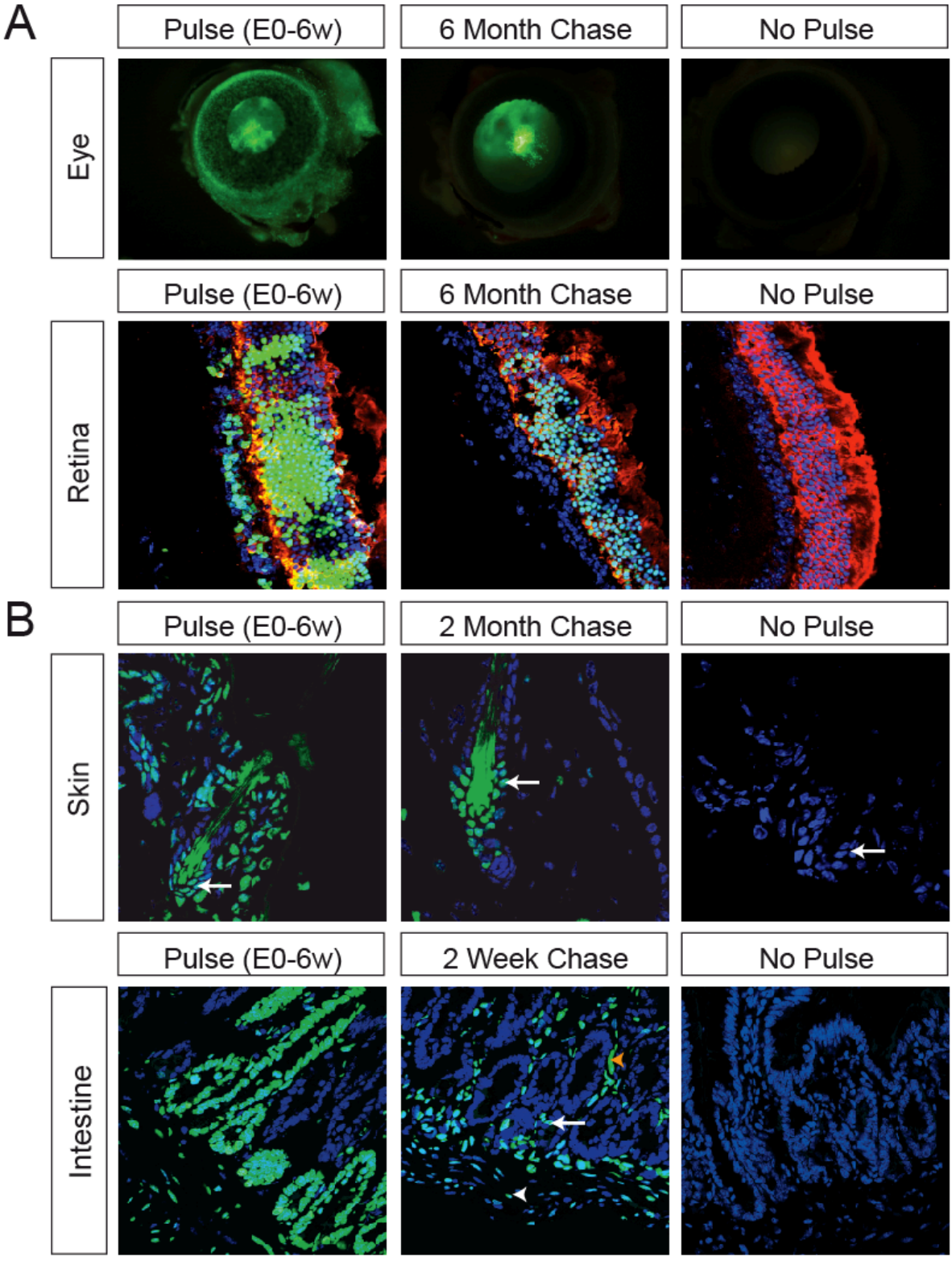
A
Pulse (E0-6w)
6 Month Chase
No Pulse
Eye
Pulse (E0-6w)
6 Month Chase
No Pulse
Retina
B
Pulse (E0-6w)
2 Month Chase
No Pulse
Skin
Pulse (E0-6w)
2 Week Chase
No Pulse
Intestine

Figure 1.3. Identification of LRCs in post-mitotic cells and stem cell populations indicates stability of H2BGFP fluorescence. A. LRCs detected in the post-mitotic photoreceptor cells of the retina after expression of Rosa26-rtTA; tetO-H2BGFP from E0-6weeks. Top panel. H2BGFP fluorescence in the eye persists at least six months following initial pulse. Magnification 20x. Bottom panel. Label retention in he retina is restricted to post-mitotic photoreceptor cells, labeled in red with anti-recoverin. Magnification 400x. All images are exposure matched. B. LRCs detected in the adult skin and intestine after expression of Rosa26-rtTA; tetO-H2BGFP from E0-6weeks. In the skin, bulge stem cells are labeled with an arrow. In the intestine, a putative crypt cell is marked with an arrow. White arrowheads mark smooth muscle and yellow arrowheads label enteric neurons. Images are exposure matched. Magnification skin 630x, intestine 400x. (Kristen Brennand, Melton lab)

No label-retaining cells are detected in the adipocyte population

To determine whether all the cells in the adipose tissue turnover at the same rate or if multiple subpopulations of cells exist, some replicative and others not, the entire pool of cells withing the adipose tissue was assayed *in vivo* for label retaining cells (LRCs). A group of 40 Rosa26-rtTA; tetO-H2BGFP animals (18 female, 22 male) were pulsed from conception until six weeks of age by administration of water containing doxycycline. Eight animals (four male, four female) were sacrificed at six weeks of age. The remaining mice were removed from doxycycline and were sacrificed after chase periods of 1 week (n=4), 2 weeks (n=4), 1 month (n=6), 2 months (n=6), 3 months (n=6) and 6 months (n=6) (Figure 1.4B). All experiments were performed on sibling cohorts. Sections of the adipose tissue were stained with Perilipin, C/EBPα (Figure 1.4B) and PECAM (Figure 1.5C) to identify fat cells and distinguish them from adipose vascular endothelium. Perilipin is a lipid-associated marker of mature differentiated adipocytes (Greenberg, A. S. et al. 1992, Tansey, J. T. et al. 2001). CCAAT/enhancer-binding protein alpha (C/EBPα) is a transcription factor that promotes adipocyte differentiation and is essential for the accumulation of lipids in adipocytes, though it is also expressed in other cell types such as myeloid lineage cells (Freytag, S. 1994, Wang, N. D. et al 1995). To verify that mature adipocytes were labeled following H2BGFP pulse, we dissociated adipose tissue from pulsed Rosa26-rtTA; tetO-H2BGFP animals and stained individual H2BGFP-positive adipocytes for Perilipin (Figure 1.5A).

Though pulse H2BGFP labeling was incomplete, in those adipocytes that were labeled, we observed uniform loss of label in adipocytes with time.

To measure the relative intensity of GFP-positive cells we used FACS analysis of adipocytes purified from the floating fraction of fat tissue. For these experiments, littermates were pulsed for at least six weeks, and chases were structured so that all animals could be sacrificed and analyzed by FACS on the same day. Importantly, comparisons of animals pulsed for 6 or 14 weeks showed no significant increase in the median intensity of the GFP+ population (data not shown). By analyzing all time points in parallel, we were able to compare the relative GFP intensity between the pulse and chase populations. This experiment was repeated three times using Rosa26-rtTA; tetO-H2BGFP mice; the results were consistent each time. FACS plots of dissociated adipocytes (Figure 1.4.C) indicated that the median intensity of the GFP fluorescence within the adipocyte pool decreased with time. As these sorts were conducted on live cells, no staining to confirm adipocyte identity was possible; however, as will be shown later, following floating purification of adipose tissue, 49% of nuclei were C/EBPα-positive (Figure 3.2), and 88% of these C/EBPα-positive nuclei were mature fat cells (Figure 3.2). This suggests that more than 40% of our floating fraction consists of mature adipocytes, which undergo near total dilution of GFP intensity within two months. No outlying population of label-retaining cells (LRCs) in the adipocyte population could be identified.

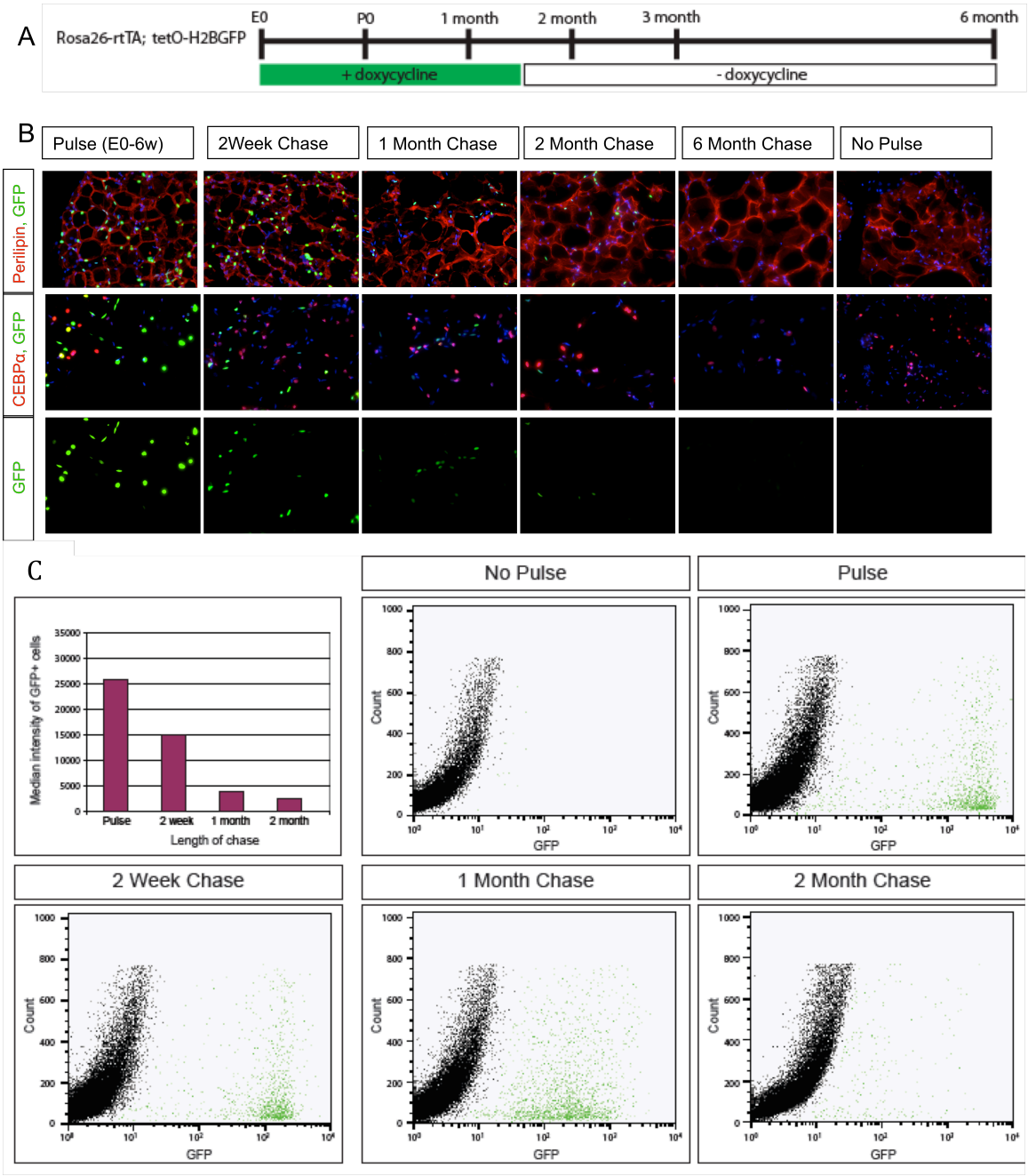

Figure 1.4. Adipocyte contribute equally to growth and maintenance

A. Timeline for Rosa26-rtTA; tetO-H2BGFP pulse-chase experiments.

B. Uniform loss of label in adipocytes following pulse-chase with Rosa26-rtTA; tetO-H2BGFP. Label retention present in the adult fat tissue (Perilipin and C/EBPα expression shown in red and DAPI stained nuclei are blue) following a chase period of up to 6 months. No adipocytes are labeled in the absence of pulse. Exposure matched images. Magnification 200x; scale bars 100μm.

E. FACS scatterplots of dissociated adipocytes of Rosa26-rtTA; tetO-H2BGFP pulse-chase animals. Scatterplot are presented as side scatter versus GFP intensity. Median intensity of GFP-positive cells versus length of chase shown in graph.

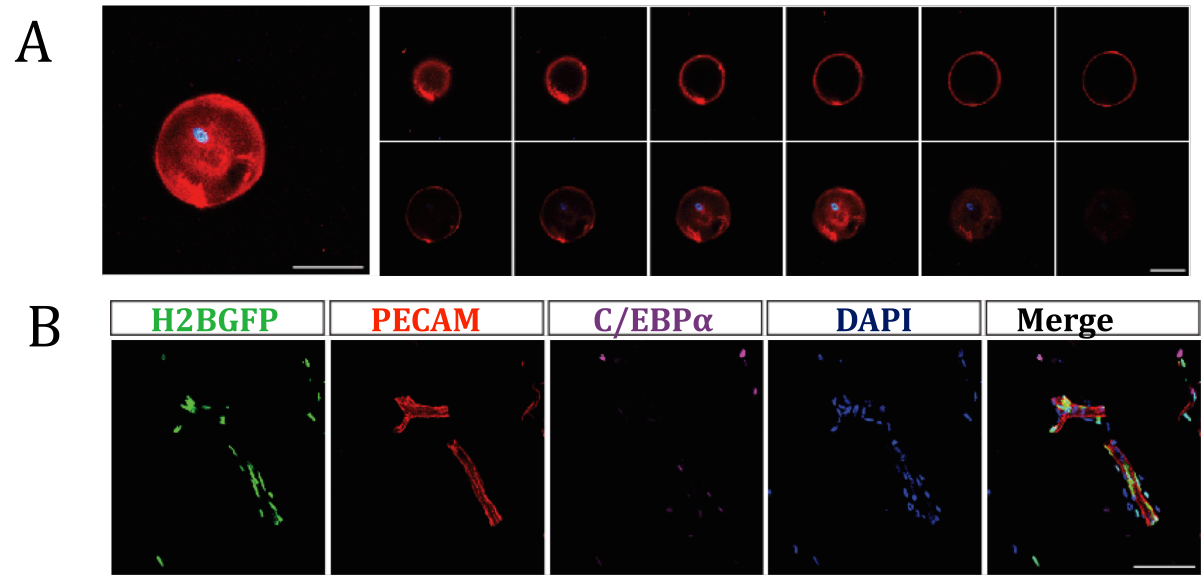

Figure 1.5. Upper panel: H2BGFP (green) is detected in the nucleus of single murine adipocytes expressing Perilipin (red). DAPI stained nuclei are blue. Magnification, 200x; scale bars 50μm.
Lower panel: coexpression of H2BGFP and PECAM indicates that Rosa26-rtTA; tetO-H2BGFP labeling marks blood vessels in addition to adipocytes, however, no overlap between the PECAM (red) and C/EBPα (purple) populations occurs in fat tissue. DAPI stained nuclei are blue. Magnification, 630x; scale bars 50μm.

Our experiments were designed to reveal whether the cells within the adipose tissue are equivalent with respect to proliferative potential and whether label-retaining cells are present within the adult adipose tissue. We examined white adipose tissue from Rosa26-rtTA; tetO-H2BGFP animals on a doxycyxline pulse–chase regime and observed a uniform loss of fluorescent signal with time (Figure 1.4B,C). Similar to results with β-cells (Brennand et al. 2007), but unlike the results in skin follicular cells, hematopoietic stem cells, muscle satellite cells and intestinal stem cells (Tumber et al. 2004, Brennand et al. 2007), no outlying population of LRCs was identified in the adipocyte population.

The diminution of H2BGFP intensity in the adipocyte population could be explained by several models. One possibility could be a high, uniform replicative rate of cell division, suggesting that all fat cell, mature adipocytes and preadipocytes, contribute equally to fat growth and maintenance in mice. If this hypothesis is correct, it would challenge the view that mature adipocytes are incapable of replication and that the cellular turnover occurring in this population of cells results entirely from preadipocyte differentiation. Another hypothesis would be that the source of loss of cells might be due to apoptosis, or macrophage engulfment or necrosis; this may be consistent with the hypothesis that new fat cells come from preadipocytes in the tissue.

No label retaining cells were detected in 1 weeks chase neonatal mice

It has been reported that a substantial expansion of adipose tissue occurs during the first postnatal month of life (G. Ailhaud 1992, Cook J 1982, Tang W, 20008). We then sought to ascertain whether the general principal of fat cell homogeneity holds true under conditions of increased cell replication during neonatal life. To address this issue, we again monitored the dilution or disappearance of the fluorescent marker H2BGFP accompanying cell division.

Adipose cells were pulse labeled with tetracycline-inducible histone 2B–green fluorescent protein (H2BGFP) (Tumbar et al., 2004) and, following a chase period, the level of fluorescence detectable within the adipose tissue was measured.

The entire neonatal adipose-cell pool was assayed for LRCs (Figure 1.6A). A group of 20 Rosa-rtTA; tetO-H2BGFP animals were pulsed until birth. Four of these animals were euthanized at birth. The remaining mice were euthanized after chase periods of 1 week (n=6), 2 weeks (n=5), and 1 month (n=5). Sections of adipose tissue were stained with Perilipin to identify fat cells. No label retaining cells were detected after 1 or 2 weeks of chase in the adipose tissue of neonatal mice. Figure 1.6B.

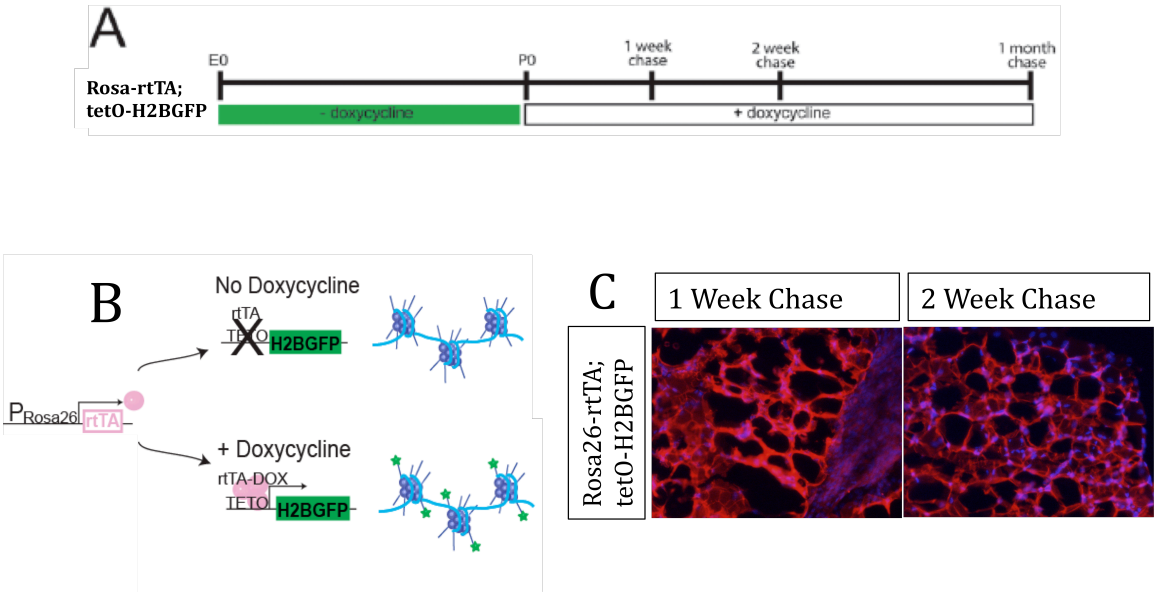

Figure 1.6. No label retaining cells in neonatal adipose tissue following pulse-chase with Rosa26-rtTA; tetO-H2BGFP. A,B Schematic of pulse-chase experiment. C. Rosa26-rtTA; tetO-H2BGFP following a chase period of 1 and 2 weeks, Perilipin is expressed in red, DAPI in blue Exposure matched images. Magnification 200x.

Due to the technical difficulty of sectioning and staining adipose tissue from newborn mice we were not able to document GFP expression in the tissue at birth. To verify that other tissues expressed GFP we analyzed islet pancreatic at birth and after chase periods of 1 week, 2 weeks and 1 month. At birth, all animals collected in the pulse group exhibited β-cells labeled with H2BGFP. We observed a uniform loss of label in β-cells over the time (data not shown, performed by Kristen Brennand, Melton lab).

BrdU ANALYSIS SUGGESTS 1% OF MURINE CELLS WITHIN THE ADIPOSE TISSUE ARE IN GROWTH PHASE ANY TIME

In order to further investigate the cellular turnover in the adipose tissue, and further substantiate the notion that cells in adipose tissue undergo frequent replication, we next assayed the ability of fat cells to incorporate an artificial nucleotide analog, 5-bromo-2'-deoxyuridine (BrdU), via DNA synthesis. Nucleotide base analogs, such as BrdU, are incorporated into DNA upon replication. Standard immunohistochemical methods can then identify the percentage of cells containing BrdU, allowing a measurement of the percentage of cells completing the S-phase of mitosis each day. By varying the duration of BrdU administration, it is possible to then plot the number of labeled cells over time and determine a rate of cellular proliferation (Figure 2.B).

The percentage of Perilipin-expressing BrdU+ cells was counted for each animal, a linear regression of the data was performed, and adipocyte BrdU incorporation in 6-week old wild-type mice was found to be 0.6% per day (n=57272 cells) (Figure 2.A; Tables 1, 5). Additionally, in a smaller population of cells, the percentage of C/EBPα-expressing BrdU+ nuclei was also determined and we observed 1.8% of C/EBPα+ cells to incorporate BrdU per day (n=5875 cells) (Figure 2.D); the increased BrdU labeling in this population may reflect BrdU incorporation by C/EBPα+ preadipocytes. It is important to note that BrdU labeling of mature adipocytes can likely result via BrdU incorporation into replicating preadipocytes immediately prior to adipocyte differentiation. No significant difference was observed between the rate of BrdU incorporation in male and female mice at 6-weeks of age (Tables 1, 5, 9);

). Thus the addition of new cells was found to be at a rate of approximately 1% per day.

To ensure that daily BrdU injection did not induce cell damage and permit replication-independent BrdU incorporation, we verified that no BrdU is detected in a post-mitotic cell population. Mammalian photoreceptors are identified by their position within the outer nuclear layer of the retina and by expression of the calcium-binding protein recoverin; there was no incorporation of BrdU by photoreceptor cells, even following 10 days of continuous BrdU injection (Figure 2.C).

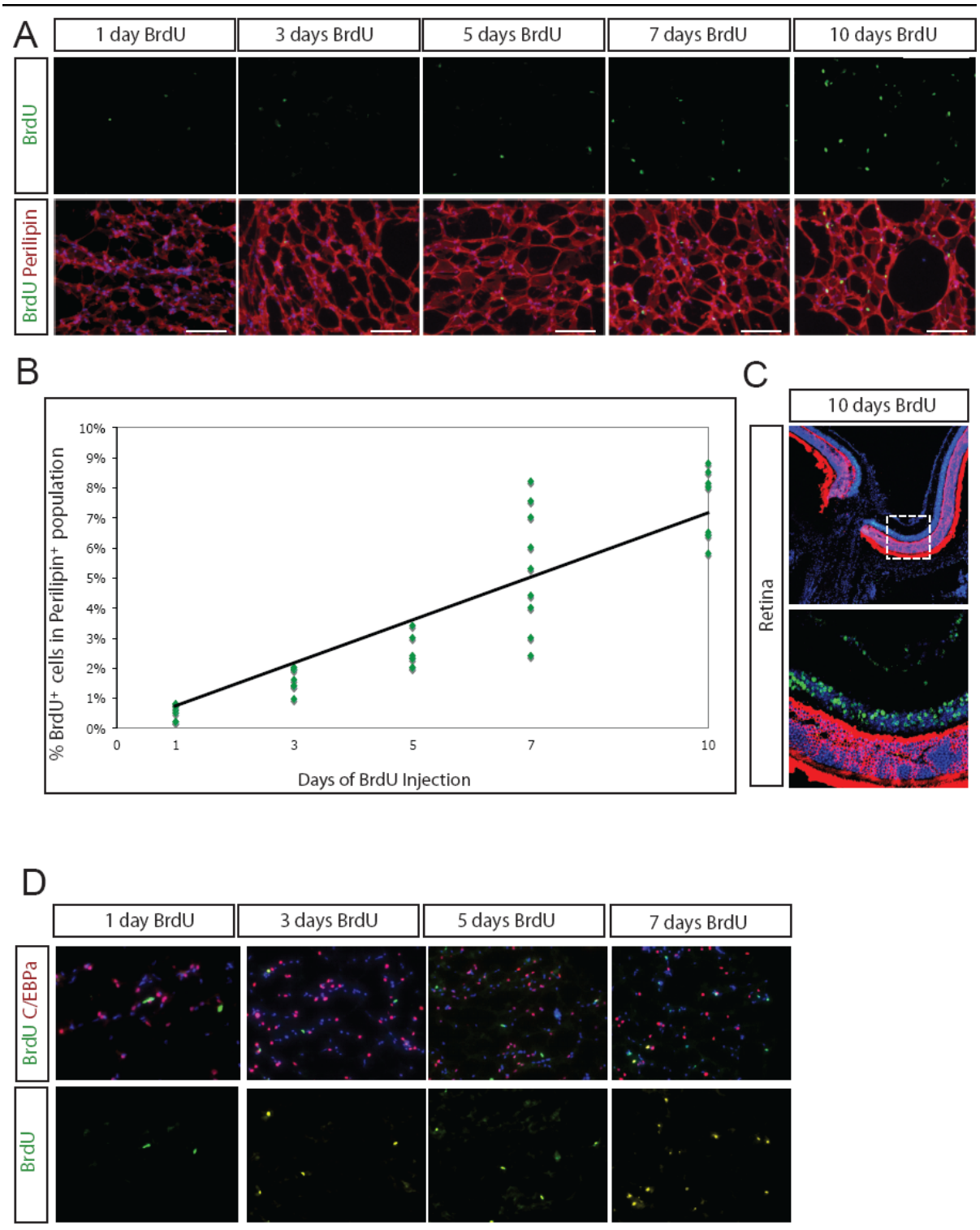

Figure 2. Adipocyte replication assayed by immunohistochemistry for BrdU incorporation. A.The rate of replication was assayed by BrdU incorporation in 6-week old C57BL/6 mice. BrdU was injected intraperitoneally daily for 1 (n=7), 3 (n=7), 5 (n=6), 7 (n=9) or 10 (n=7) continuous days. Perilipin staining is shown in red, BrdU staining in green, DAPI stained nuclei are blue. Magnification, 200x; scale bars 100μm.

B. Linear regression analysis of BrdU counts. Each data point on the graph represents the percentage of BrdU-positive cells in the Perilipin-positive population from one animal. Rate of adipocyte replication in 6-week old mice is 0.6% per day.
C. No BrdU is detected in post-mitotic photoreceptor cells. Recoverin, a marker of photoreceptor cells, is shown in red, BrdU staining in green, DAPI stained nuclei are blue. Top panel, 100x. Bottom panel, 400x. Bottom panel represents the region within the dashed line in the top panel.
D. The rate of replication was assayed by BrdU incorporation in 6-week old C57BL/6 mice. BrdU was injected intraperitoneally daily for 1, 5 , or 7 continuous days. C/EBPα staining is shown in red, BrdU staining in green, DAPI stained nuclei are blue. Magnification, 200x.

APPROXIMATELY 5% OF C/EBPα POSITIVE CELLS IN ADIPOSE TISSUE ARE IN THE CELL CYCLE ANY TIME

To confirm our results showing replication in fat tissue, we assessed a known marker of cell division within the adipose population. The Ki67 antigen is present only in actively dividing cells and is a robust marker of cell replication. It is a nuclear protein found during G1 and S/G2/M phases of the cell cycle but not detectable in quiescent G0 cells (Lalor, P. A et al., 1987). Although the biological function of the antigen remains unresolved, the antigen is essential for maintaining the proliferating state of cells (Sasaki, K 1987). We used immunohistochemistry to quantify the percentage of fat cells, identified by nuclear expression localiztion of C/EBPα, expressing Ki67. Within the C/EBPα-positive population, some nuclei were strongly labeled and others dimly labeled; we counted all labeled nuclei as positive. We determined the percentage of C/EBPα-positive adipocytes in cell cycle in C57/BL6 mice at various stages (Figure 3.1 A,B; Tables 2, 5). At 6-, 14-, and 30-weeks of age, there was no significant difference in the percentage of C/EBPα-positive, Ki67-positive cells in male and female adipose tissue (Tables 2, 5, 6, 9). We found that 49% of the nuclei in adipose tissue were C/EBPα-positive and that 5% of C/EBPα-positive cells expressed Ki67 at any given point in time (Figure 3.1 B; Table 2).

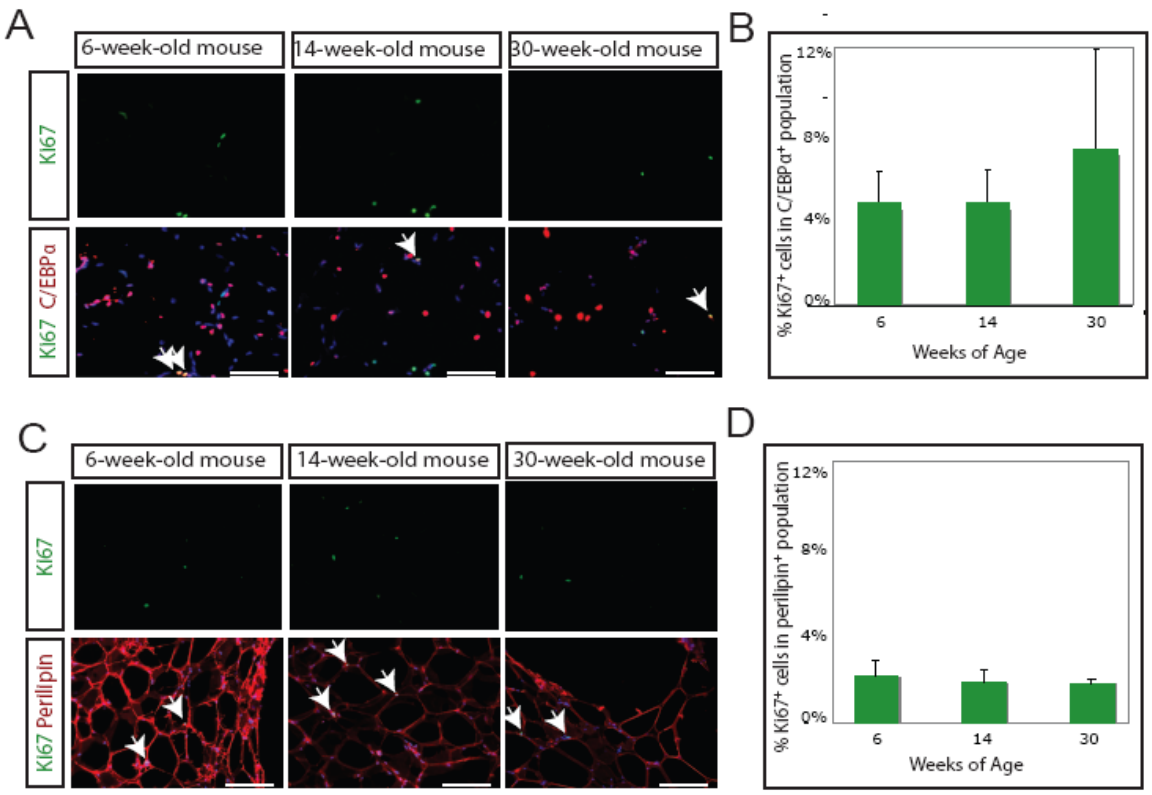

Figure 3.1. Adipose cells replication assayed with the cell cycle protein Ki67.

A. The rate of replication in the adipose tissue was assayed by co-staining for the cell cycle protein Ki67 (green) and the adipocyte marker C/EBPα (red) in mice. Representative images of 6-week-old, 14-week-old and 30-week-old stained adipose tissue are shown. White arrows indicate cells co-staining for Ki67 and C/EBPα. DAPI stained nuclei are blue. Magnification, 200x; scale bars 100μm.

B. The percentage of C/EBPα-expressing cells in the growth cycle was quantitated in 6 (n=6), 14 (n=9), and 30-week old (n=3) C57/BL6 mice. We find that throughout adolescence and adult life, 4.8% of adipocytes are Ki67 and C/EBPα-positive at any time. Error bars indicate standard deviation.

C. The rate of cells replication was assayed by co-staining for the cell cycle protein Ki67 (green) and the adipocyte marker Perilipin (red) in mice. Representative images of 6-week-old, 14-week-old and 30-week-old stained adipose tissue are shown. DAPI stained nuclei are blue. Magnification, 200x; scale bars 100μm.

D. The percentage of cells, in the growth cycle, within the Perilipin cells was quantitated in 6- (n=6), 14- (n=7), and 30- (n=6) week-old C57/BL6 mice. Approximately 1.8% of the cells were found Ki67 positive at any time. Error bars indicate standard deviation.

To investigate the presence of Ki67-positive cells in the adipocyte population, we used FACS analysis of purified adipocytes from six 8-week-old and six 10-week-old mice. Due to the presence of large lipid droplets, adipocytes are extremely buoyant and can be easily separated from the other cell types present in dissociated fat tissue based on this physical attribute (Tang et al. 2008, Zhang, H. H 2000). Adipocytes were purified from the non-floating stromal/vascular (SV) fraction of fat tissue; the nuclei were extracted from adipocytes and SV cells, followed by staining for C/EBPα, Ki67 and the nuclear marker DAPI. The total percentage of Ki67-positive, C/EBPα-positive nuclei in the floating fat sample was as high as 4.09% (Figure 3.2A). Importantly, to verify that the majority of C/EBPα expression in the floating fraction occurred in adipocytes, floated and collagen embedded fat cells were subjected to immunohistochemistry, thereby determining the percentage of C/EBPα-positive cells that were positive for the mature fat markers Perilipin or the fluorescent fatty acid analog BODIPY. We find that 88% of C/EBPα-positive cells in the floating population are mature fat cells (Table 14). The identity of the remaining 12% of C/EBPα-positive nuclei remains unknown; they may represent preadipocytes or myeloid contaminants. However, we observed a population of C/EBPα-high, Ki67-positive cells present to a greater extent in the floating fraction than the SV fraction (Figure 3.2), suggesting the possibility of a proliferative population in mature fat cells. Taken together, the detection of Ki67-positive C/EBPα-positive nuclei in the floating fraction of dissociated fat open the possibility that that lipid-containing, C/EBPα-expressing adipocytes are capable of replication.

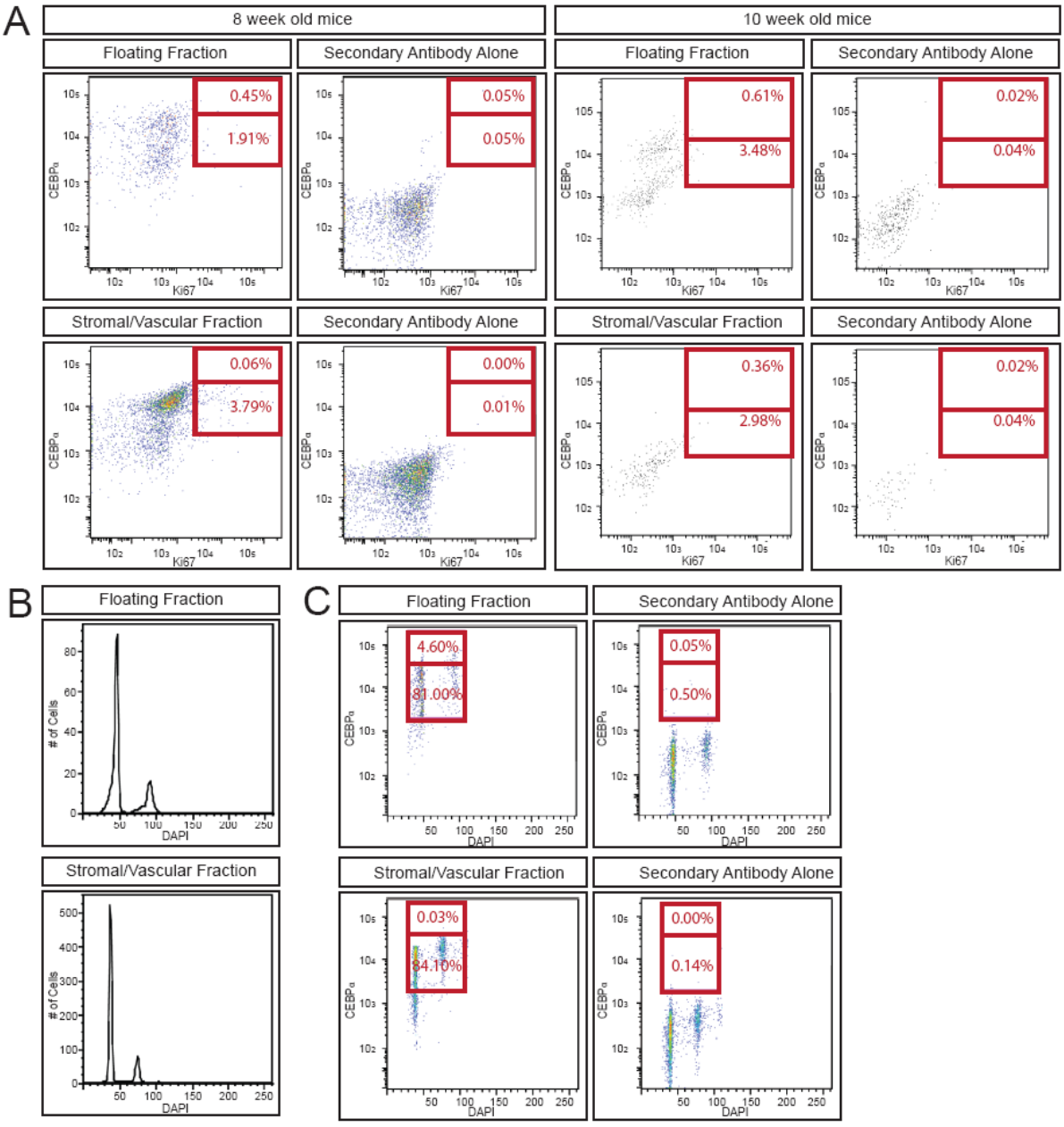

Figure 3.2. FACS analysis of adipocyte replication with the cell cycle protein Ki67.
A. Top, FACS plots of the floating adipocyte nuclei from dissociated fat tissue of wild-type mice, stained for Ki67 and C/EBPα, as well as appropriate secondary antibody controls. The total percentage of Ki67-positive C/EBPα-positive nuclei in the floating fat sample (above secondary alone background for all antibodies) was 2.36%; of this 0.45% represented C/EBPα-high Ki67-positive events, and 1.91% represented C/EBPα-low Ki67-positive events. Bottom, FACS plots of the stromal/vascular nuclei from dissociated fat tissue of wild-type mice, stained for Ki67 and C/EBPα, as well as appropriate secondary antibody controls. The total percentage of Ki67-positive C/EBPα-positive nuclei in the stromal/vascular fat sample (above secondary alone background for all antibodies) was 3.85%; of this 0.06% represented C/EBPα-high Ki67-

positive events, and 3.79% represented C/EBPα-low Ki67-positive events. Left panels represent 8-week-old mice, right panels represent 10-week-old mice.
B. A histogram representing the distribution of DAPI staining within floating (top) and the SV (bottom) adipocyte fractions shows a secondary peak of potentially 4n cells undergoing replication.
C. Top, 85.60% of the nuclei in the floating adipocyte fraction stain positive for C/EBPα by FACS analysis; 4.60% stain as a C/EBPα-high population not present in the stromal/vascular fraction. Bottom, 84.13% of the nuclei in the stromal/vascular fraction stain positive for C/EBPα by FACS analysis, but only 0.03% stain as a C/EBPα-high population.

However, it is very important to notice that the analysis of adipose tissue by immunohistochemistry is quite challenging experimentally. Adipose tissue is highly vascularized and every adipocyte is juxtaposed with multiple capillaries [Crandall et al., 1997], making it very difficult to determine if a nuclear antigen such as Ki67 is a present in the nucleus of an adipocyte or in the nucleus of an adjacent endothelial cell by conventional microscopy (Figure 3.3). In addition, macrophage infiltration of fat tissue may occur, adding further complexity by introducing yet another type of cell that is difficult to separate from adipocytes by analysis using conventional microscopy. In addition, macrophages are known to engulf debris such as dying cells by phagocytosis, leading to an additional potential source of error. To circumvent these problems and definitively identify Ki67-positive fat cells, it would be ideal to perform stainings with a nuclear marker of mature adipocytes. However, to our knowledge no nuclear marker for mature adipocytes has been identified. C/EBPα exhibits nuclear localization, but is expressed in both preadipocytes and mature fat cells, whereas Perilipin, a lipid-associated marker of mature differentiated adipocyte, is found associated with the lipid membrane surrounded the lipid droplet and it is the confined to the cytoplasm. Other markers for mature adipocytes such as adipsin, a serine protease homolog synthesized and secreted by adipose cells (Wilkison, W. O. 1990, Cook, K. S. et

al. 1987), also exhibit cytoplasmic localization, and consequently do not demarcate which nuclei belongs to a mature fat cell (Figure 3.3).

To further examine the identity of Ki67-positive cells in adipose tissue, we analyzed stained sections of whole-mount fat tissue by confocal microscopy. Sections of whole-mount adipose tissue were subjected to immunohistochemistry using antibodies against perilipin, Ki67, and PECAM (CD31), which is a well-accepted marker of endothelial cells (Newman, P. J. et al. 1994) (Figure 3.6 A.B, Table 3). Confocal microscopy revealed that 0.8% of all cells were positive for both Ki67 and PECAM. For 1.6% of the total number of Ki67 positive cells a definitive classification could not be determined, due to either a lack of staining for both PECAM and Perilipin, or because of overlapping immunoreactivity for both Perilipin and PECAM. 1.8 % of the nuclei positive for Ki67 were negative for PECAM and they appeared to be located within perilipin-expressing cells; however, even after performing Z-stack analysis, we could not with absolute certainty exclude the possibility that these nuclei belonged to preadipocytes or macrophages. (Figure 3.4) We investigated the presence and replication of macrophages within adipose tissue in an attempt to further clarify this issue. In some cases we failed to determine whether a cell was a macrophage or adipocyte expressing Perilipin, but there was frequently no co-labeling of the macrophage marker Mac1 with Perilipin (Figure 3.5B) or strong C/EBPα expression (Figure3.5C).

We stained whole-mount sections of adipose tissue with Ki67 and the macrophage marker Mac1 in 6 and 14 week-old C57/BL6 mice. The percentage of Ki67, Mac1 double positive cells (Figure 3.6 C,D and table) was found to be 0.7 % . We then determined the percentage of adipose Ki67 expressing cells on whole-mount sections stained with Perilipin in C57/BL6 mice at various ages (Figure 3.1E, F; Table 4). We found that throughout adult life, 1.8% of cells within the Perilipin populaiton were positive for Ki67 at any given time (Figure 3.1F; Tables 4, 5, 6). These results are in keeping with our previous count of

replicating cells in whole-mount adipose sections stained with Ki67, Perilipin and CD31 (Figure 3.6). However, it is important to remember that this cannot be considered definitive proof of mature adipocyte replication due to the absence of a nuclear marker for mature adipocyte.

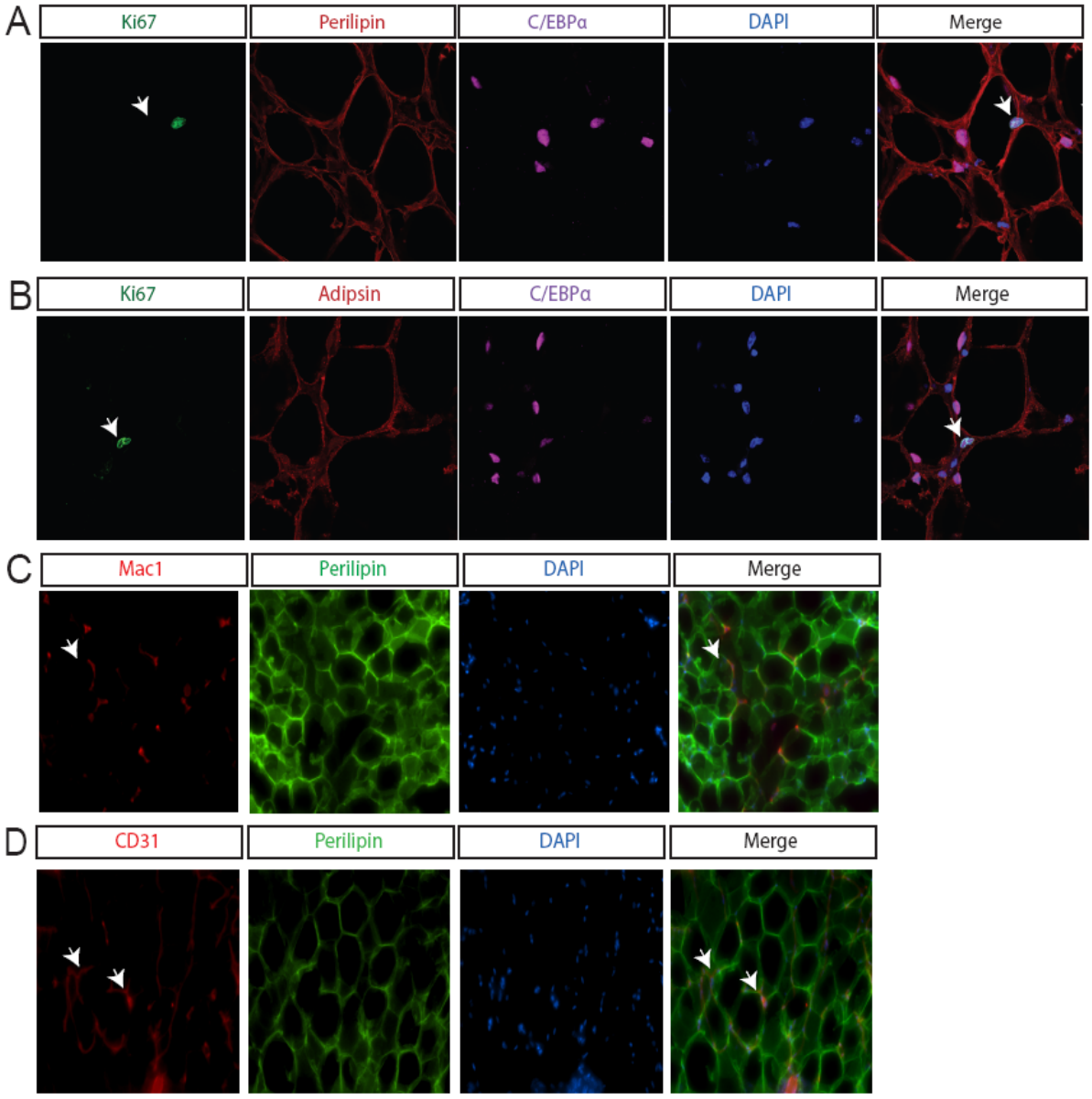

Figure 3. 3 Technical difficulties distinguishing cells within adipose tissue
A. Ki67 (green) overlaps with C/EBP/α (purple) in adipose tissue positive, DAPI stained nuclei are blue and Perilin is in red. White arrow indicate nuclei double positive for Ki67 and C/EBP/α surrounded by Perilin staining. The imagine shows the difficulty of determining

whether the double positive nuclei belong to a mature fat cell stained with Perilipin (red) or a preadipocyte or macrophages. Magnification, 630x; scale bars 50μm.
B. Ki67 (green) overlaps with C/EBPα (purple) in adipose tissue. White arrow indicates double positive nuclei. DAPI stained nuclei are blue. The imagine shows the difficulty of determining whether the double positive nuclei belong to a mature fat cell stained with Adipsin (red) or a preadipocyte or macrophages. Magnification, 630x; scale bars 50μm.
C. Section of murine adipose tissue taken with the conventional microscope. White arrow show that the vascular endothelial marker CD31 (green) can overlap with the adipocyte maker Perilipin (red) in adipose tissue. DAPI stained nuclei are blue. Magnification, 200x; scale bars 100μm.
D. With conventional microscope the macrophage marker Mac1 (green) can overlap with the adipocyte maker Perilipin (red) in adipose tissue. DAPI stained nuclei are blue. Magnification, 200x; scale bars 100μm.

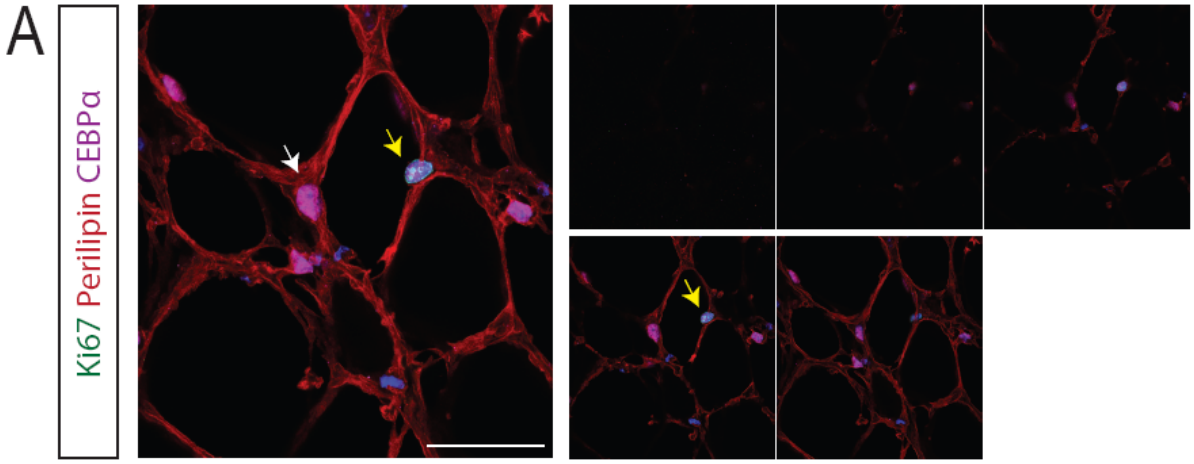

Figure 3.4. Confocal microscopy z-stack images of murine adipose cells
A.Confocal z-stack images showing a double positive nuclei for the cell cycle protein Ki67 (green) and C/EBPα (purple) *in situ* (yellow arrow). Perilipin in red. (see figure 3.3A for single channel).White arrows indicate a C/EBPα positive nucleus. Due to the particular structure of the adipose tissue, z-stack doesn't allow us to confirm whether the Ki67,C/EBPα posive nucleus belong to a mature fat cells or preadipocytes or macrophages. DAPI stained nuclei are blue. Magnification, 630x; scale bars 50μm.

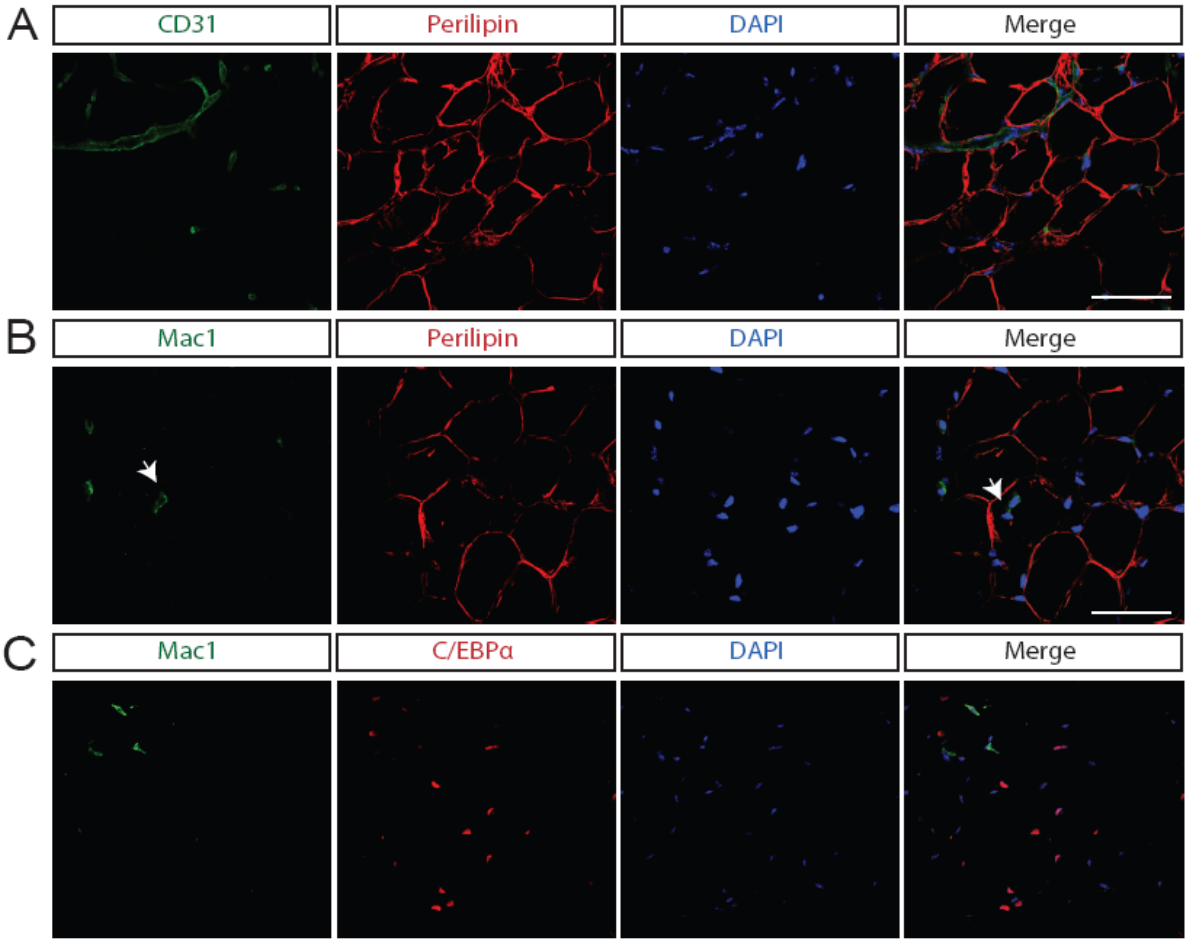

Figure 3.5 Confocal microscopy images in adipose tissue.

A. Confocal microscopy may allow to distinguish the vascular endothelial cells stained with CD31 (green) and adipocytes stained with Perilipin (red) in adipose tissue. DAPI stained nuclei are blue. Magnification, 200x; scale bars 100μm.

B. The macrophage marker Mac1 (green) does not overlap (white arrow) with the adipocyte maker Perilipin (red) in adipose tissue. DAPI stained nuclei are blue. Magnification, 200x; scale bars 100μm.

C. The macrophage marker Mac1 (green) is not coexpressed in those cells strongly expressing C/EBPα (red) in adipose tissue. DAPI stained nuclei are blue. Magnification, 200x; scale bars 100μm.

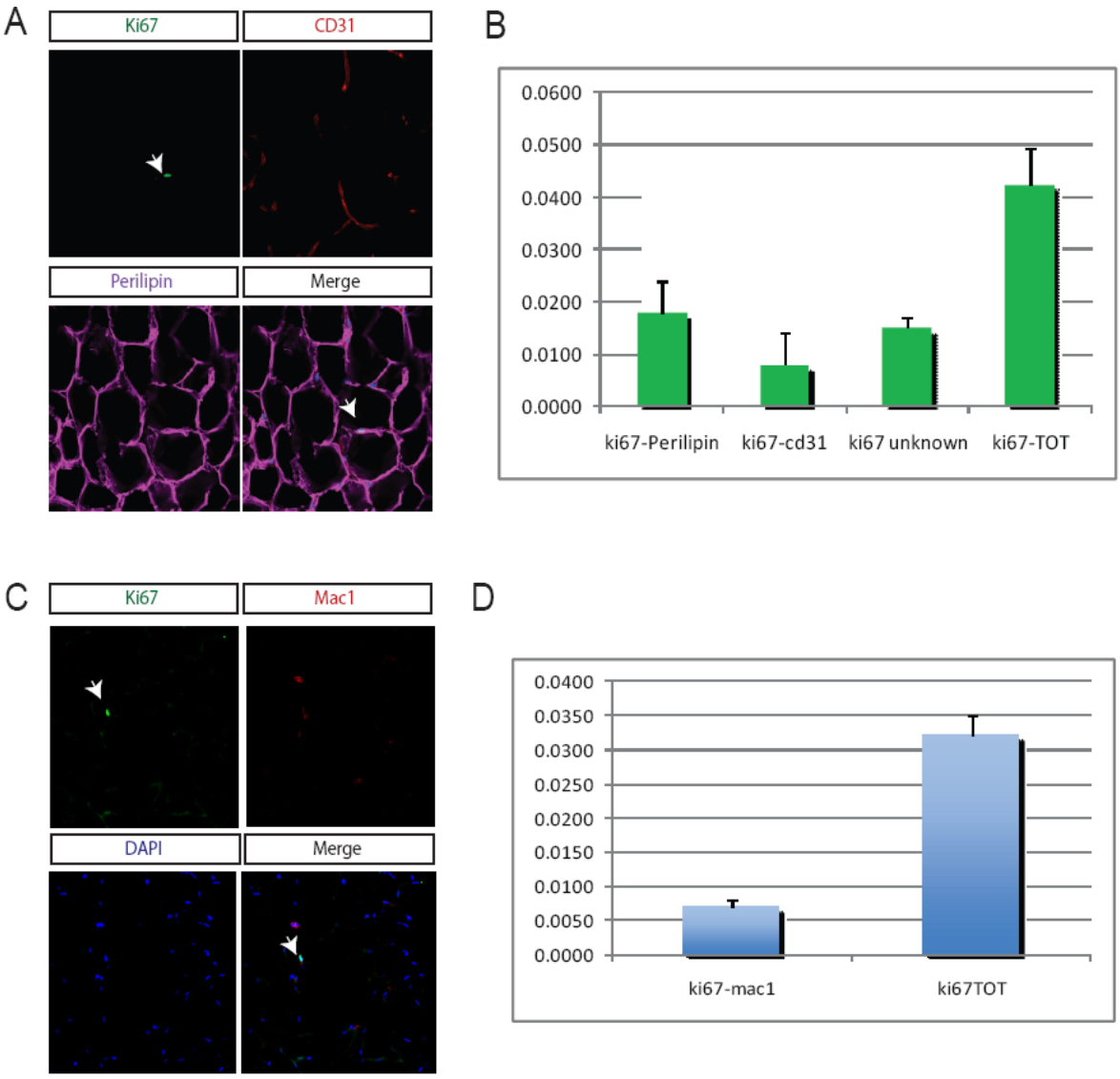

Figure 3.6. Adipocyte replication assayed with the cell cycle protein Ki67.

A. The rate of adipose cells replication was assayed by co-staining for the cell cycle protein Ki67 (green), the adipocyte marker Perilipin (purple) and the vascular marker CD31 in mice. Representative images of 6-week-old stained adipose tissue are shown. White arrows indicate cells positive for Ki67 and negative for CD31, located within Perilipin postive cells. DAPI stained nuclei are blue. Magnification, 200x; scale bars 100μm.

B. The percentage of adipose cells in the cell cycle was quantitated in 6 (n=2), 14 (n=1) week-old C57/BL6 mice. We found 0.8% of cells Ki67-cd31 positive and Perilipin negative; 1.8% of cells Ki67 positive-cd31 negative within Perilipin expressing cells and for 1.5% of the cells it was not possible determine the identity. Error bars indicate standard deviation.

E. The rate of adipose tissue replication was assayed by co-staining for the cell cycle protein Ki67 (green) and the macrophage marker Mac1(red) in mice. Representative images of 6-week-old, stained adipose tissue are shown. White arrows indicate cells co-staining for Ki67 and Mac1. DAPI stained nuclei are blue. Magnification, 200x; scale bars 100μm.

D. The percentage of mac1 expressing cells in the growth cycle was quantitated in 6 (n=1), 14-(n=1), C57/BL6 mice. Approximately 0.7% of macrophages are Ki67 positive at any time. Error bars indicate standard deviation.

In summary, we investigated the percentage of Ki67 positive cells in the adipose tissue of C57/BL6 mice at various ages (Figure 3.1; Table 2, 3, 4, 5, 6). Whole-mount adipose tissue analysis performed using conventional microscopy showed that 49% of the nuclei in adipose tissue were C/EBPα-positive and that 4.8% of C/EBPα-positive cells were in the cell cycle at any given point in time (Figure 3.1A; Table 2). FACS analysis of nuclei from dissociated fat tissue of 8 weeks old wild-type mice stained for Ki67 and C/EBPα showed that the total percentage of Ki67-positive C/EBPα-positive nuclei in the floating fat sample was 2.36%; of this 0.45% represented C/EBPα-high Ki67-positive events. FACS plots of the stromal/vascular nuclei from dissociated fat tissue of wild-type mice, stained for Ki67 and C/EBPα, showed that the total percentage of Ki67-positive C/EBPα-positive nuclei was 3.85%; of this 0.06% represented C/EBPα-high Ki67-positive events, and 3.79% represented C/EBPα-low Ki67-positive events. In addition, performing confocal analysis on whole-mount adipose tissue of 3 animals, we counted a total of 4.2% of Ki67 positive nuclei: 0.8% represented CD31 positive Ki67 positive cells, 1.8% were Ki67 positive cells negative for CD31 exhibiting overlapping immunoreacitivity with Perilipin, while for 1.5% of Ki67 positive cells we could not determine the nature of the cellular type.

Our experiments allowed us to estimate the turnover rate of cells in adipose tissue based on the addition of new cells measured by BrdU incorporation and loss of cells measured by the loss of H2B-GFP in the tissue.

Our results lead us to advance two hypothesis:

1)We could hypothesize that the adipocytes might be lost due to apoptosis or macrophage engulfment. Thus we could hypothesize that new fat cells come from preadipocytes in the tissue. A wealth of previously published data show that adipocyte precursors are capable of both maintaining the progenitor pool and producing adipocytes, and indeed, we found evidence of replication in the SV fraction of adipose tissue in all of our experiments.

2)If cells would not be lost through apotosis or macrophage engulfment or necrosis, we could hypothesize that mature adipocyte may undergo replication, and contribute to maintaining and expanding the adipocyte population. However, it is important to remember that our data suggesting fat cells replication can't be considered definitive proof of mature adipocyte replication due to the absence of a nuclear marker for mature adipocyte. We then aimed to investigate this second hypothesis by using lineage experiments discussed below.

LINEAGE-TRACING EXPERIMENTS TO INVESTIGATE WHETHER NEW ADIPOCYTES ARISE FROM EXISTING ADIPOCYTES

To directly compare the replication capacity of adipose cells, we performed a lineage-tracing analysis of adipose tissue. AP2-CreER transgenic mice (Imai, T. et al. 2001, Dali-Youcef, N. et al. 2007) drove expression of tamoxifen-dependent Cre recombinase specifically in adipocytes, while a floxed-stop-LacZ reporter system driven from the Rosa26 locus (Hayashi, S. & McMahon, A. P. 2007) was used to irreversibly label cells with Cre recombinase expression. In this case, a pulse of tamoxifen induced permanent and heritable labeling of the adipocyte population. Following a chase period sufficiently long to permit the turnover and replacement of most fat cells, two possible outcomes exist. If a substantial loss of lacZ label occurred, this would indicate that a population of stem or progenitor cells mediates adipocyte turnover. Conversely, the persistence of most lacZ label in the adipocyte population would be consistent with our second hypothesis that adipocytes are capable of replication, thereby maintaining the adipocyte population (Figure 4.1A).

We injected tamoxifen (3 mg tamoxifen per 40 g weight) daily for 5 days into 10 AP2-CreER; R26R mice at 6 weeks of age. Five animals were sacrificed approximately 72 hours after tamoxifen injection (the pulse group), and we observed substantial adipocyte labeling in both whole-mount and sectioned tissue (Figure 4.1A, B). We sacrificed the remaining mice after a chase period of 2 months and compared the lacZ label in adipose tissue of pulse and chase mice at this time. It was extremely difficult to determine the percentage of labeled adipocytes *in vivo* as lacZ staining was strong and diffuse throughout the cytoplasm. Instead, to quantitate the lacZ labeling in adipose tissue, 115 images from pulse mice and 124 images from chase mice were processed to

select the non-cellular portion of the images and create two new images from each: the first containing only the blue pixels from the image (representing the lacZ stained cellular material), and the second containing only the non-blue pixels from the image (Tolivia, J. et al. 2006, Lehr, H. A. et al. 1999). The total number of pixels in each image was determined and the proportion of blue pixels was calculated as a representation of total lacZ staining in the adipose tissue.

We found that 40% of tissue pixels were labeled with lacZ following the initial tamoxifen pulse and that 42% of tissue pixels were labeled with lacZ following a two month chase (Figure 4.1C; Table 15). This persistence of lacZ labeling in the fat cell population may be interpreted as support for adipocyte replication, suggesting that all adipocytes contribute equally to fat growth and maintenance in wild-type mice by undergoing replication and cell division.

One potential caveat to this experiment would be the possibility of "leaky", ongoing recombination, which would make results very difficult to interpret. To verify that the AP2CreER is not leaky we stained adipose tissue of AP2CreER;R26R-lox-stop-lox-LacZ mice not treated with tamoxifen (Figure 4.2A). As expected, no lacZ labeling was seen in the absence of tamoxifen.

To test whether fat cells express beta galactosidase, we stained sections of adipose tissue of AP2CreER;R26R-lox-stop-lox-LacZ with anti-Cre antibody (Figure 4.2B).

It was important to verify that AP2-CreER labeling occurred specifically in adipocytes. We separated the floating fraction of adipocytes from the SV fraction and determined the percentage of lacZ-labeled cells in the SV population through two independent methods. First, we plated the SV fraction, cultured it to subconfluence *in vitro*, and then counted the percentage of lacZ-positive cells in the total DAPI-positive population. 1.9% of labeling was observed in the SV fraction of AP2-CreER;R26R mice in the presence of tamoxifen, which is comparable to the percentage of SV cell labeling observed

in tamoxifen injected control mice (1.2%) (Figure 4.1D; Table 16). Second, we embedded individual SV cells in collagen and counted the percentage of lacZ-positive cells in the total DAPI-positive population. 1.6% was observed in the stromal vascular fraction of AP2-CreER;R26R mice in the presence of tamoxifen, which is comparable to the percentage of stromal vascular cell labeling observed in tamoxifen injected control mice (0.4%) (Figure 4.1D; Table 17). Given that AP2-CreER;R26R-mediated labeling of nonadipocytes is low, this genetic system appears to be an effective tool permitting permanent labeling of mature adipocytes.

These experiments were designed to provide genetic evidence that mature adipocytes give rise to new adipocytes. We examined white adipose tissue from AP2-CreER;R26R pulse–chase animals and did not observe loss of lacZ label with time (Figure 4.1C). Therefore, though sufficient turnover of the adipocyte population occurs within two months to dilute the H2BGFP label *in vivo*, our experiments may suggest that one source of these new adipocytes may be preexisting adipocytes, as evidenced by the permanent, heritable lacZ labeling within fat cells. According to this model, the adipocyte population would be maintained at least in part by adipocyte replication.

However, it is important to notice that the 1.9% of the SV fraction positive for LacZ, could represent a population of precursors which proliferate to give rise to a substantial proportion of adipocytes. Further experiments are required to definitively determine whether and how the total number of adipocytes is preserved.

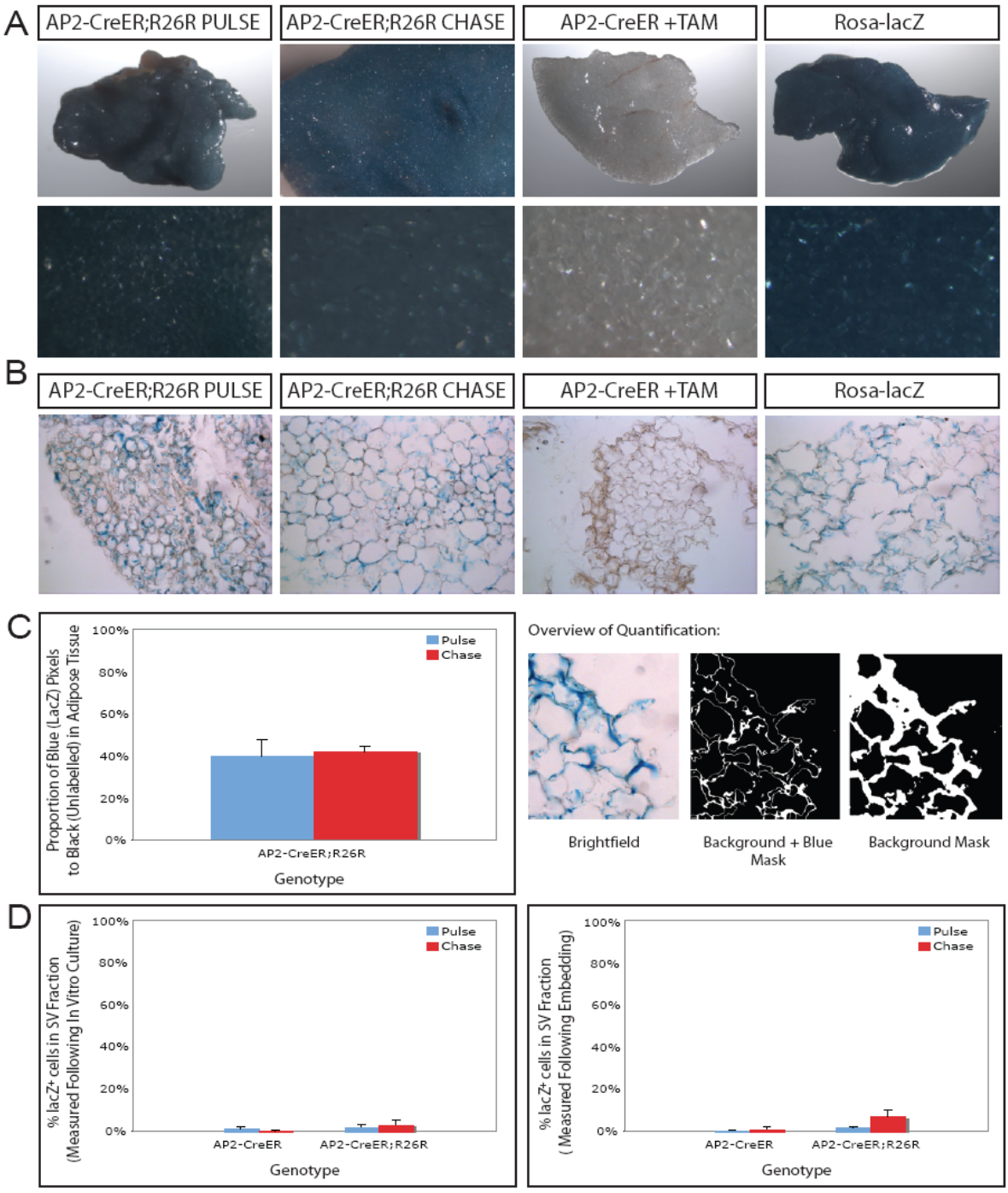

Figure 4.1. Lineage analysis of adipose tissue using tamoxifen inducible AP2-CreER labeling in adult mice.

A. By whole-mount analysis, uniform lacZ staining is observed in both pulse and 2 month chase adipose tissue in AP2-CreER;R26R mice. Very minimal lacZ expression is detectable in tamoxifen injected R26R mice. Strong adipose labeling is observed when the native Rosa26 promoter drives lacZ expression.

B. In sectioned tissue, uniform lacZ staining is observed in both pulse and 2 month chase of adipose tissue in AP2-CreER;R26R mice. Very minimal lacZ expression is detectable in tamoxifen injected R26R mice. Strong adipose labeling is observed when the native Rosa26 promoter drives lacZ expression

C. LacZ staining in Ap2-CreER; R26R pulse and 2 month chase animals is virtually unchanged. 40% of tissue pixels were labeled with lacZ following the initial tamoxifen pulse and 42% of tissue pixels were labeled with lacZ following a two month chase. To quantitate the lacZ labeling in adipose tissue, brightfield images were semi-automatically processed to select the non-cellular portion of the images, after which the script created two images either containing (background + blue mask) or not containing (background mask) the blue pixels from the image. Error bars indicate standard deviation.

D. When measured following purification and *in vitro* culture (left), only minor labeling (1.91+/-1.40%) was observed in the stromal vascular fraction of Ap2-CreER; R26R mice following a pulse of tamoxifen, which is extremely comparable to the percentage of stromal vascular cell labeling observed in tamoxifen injected R26R mice (1.17+/- 1.07%). When measured following purification and collagen embedding, (right), only minor labeling (1.64+/-0.50%) was observed in the stromal vascular fraction of Ap2-CreER; R26R mice following a pulse of tamoxifen, which is extremely comparable to the percentage of stromal vascular cell labeling observed in tamoxifen injected R26R mice 0.38+/-0.42%). Error bars indicate standard deviation.

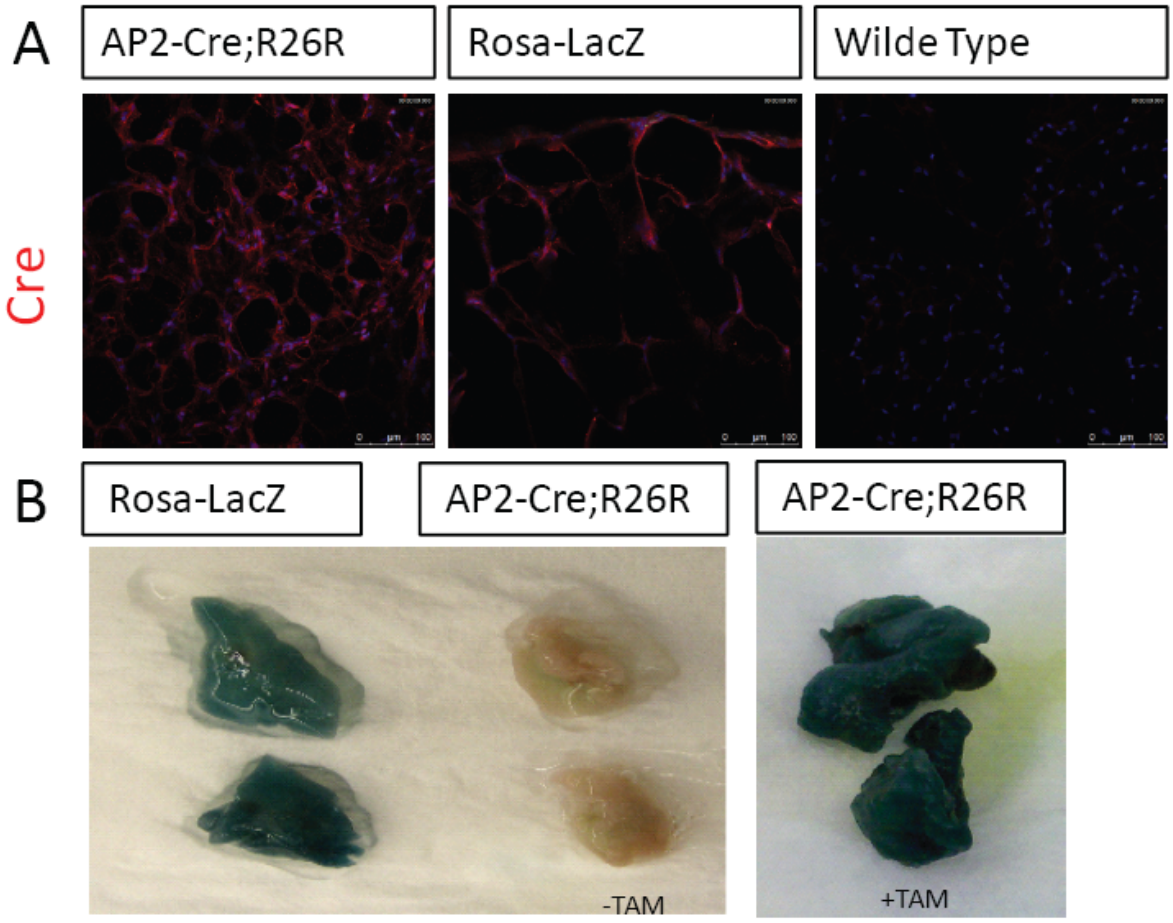

Figure 4.2. AP2-CreER;R26R mice express Cre in WAT and and don't show leaky ongoing recombination A. Adipose tissue sections of AP2-CreER;R26R, Rosa-LacZ mice and wild type mice stained with anti-Cre antibody (red).

B. By whole-mount analysis, uniform lacZ staining is observed in adipose tissue in AP2-CreER;R26R mice treated with tamoxifen and in constitutive LacZ mice. No LacZ stain is detected in AP2-CreER;R26R mice not treated with tamoxifen.

ADIPOSE TISSUE REPLICATION IN PARTICULAR CONDITIONS

Obese mice exhibit increased levels of adipose tissue replication

We next sought to determine whether the rate of adipose cells replication is altered in obese mice. To do this, we utilized OB/OB mutant mice, in which a mutation in the leptin gene results in profound obesity (Friedman, J. M. et al 1999). Leptin is a hormone expressed predominantly in adipose tissue to modulate body weight and energy expenditure (Halaas, J. L. et al. 1997); it is thought to be expressed only in terminally differentiated adipose cells (Leroy, P. et al. 1996), where its expression is regulated by C/EBPα (Miller, S. G. et al. 1996).

To address whether adipose tissue replication is altered in obesity, we determined the percentage of C/EBPα positive cells in the cell cycle in 14-week-old wildtype (n=9) and OB/OB (n=6) mice, all of which were maintained on a C57/BL6 background (Tables 2, 7). Ki67 and C/EBPα co-expressing fat nuclei were counted for both the wildtype and OB/OB groups, and found to be 4.6% and 7.3%, respectively (Figure 5.1A,B). This represents a statistically significant increase in adipose tissue replication in OB/OB mice ($p<0.05$) (Table 9). Within either the wildtype or OB/OB groups, no significant difference was observed between adipose tissue replication in male and female mice (Tables 2, 5, 6, 7). To further characterize the increase in adipose cell replication in OB/OB mice, 14-week-old wildtype (n=5) and OB/OB (n=6) mice were injected with BrdU daily for 5 days (Table 8). BrdU and C/EBPα co-labeled adipose nuclei were counted for both groups, and BrdU incorporation was found to undergo a significant increase ($p<0.001$), from 2.9% to 9.7%, in OB/OB mice (Figure 5.1C, D; Tables 8, 9). We then determined the percentage of adipose cells in the cell cycle in 6-week-old OB/OB (n=3) and 14-week-old OB/OB (n=1) mice. We counted the percentage of Ki67, C/EBPα

within Perilipin expressing cells and found it to be 2.4% and 10.4%, respectively (Figure 5.2 and Table 7).

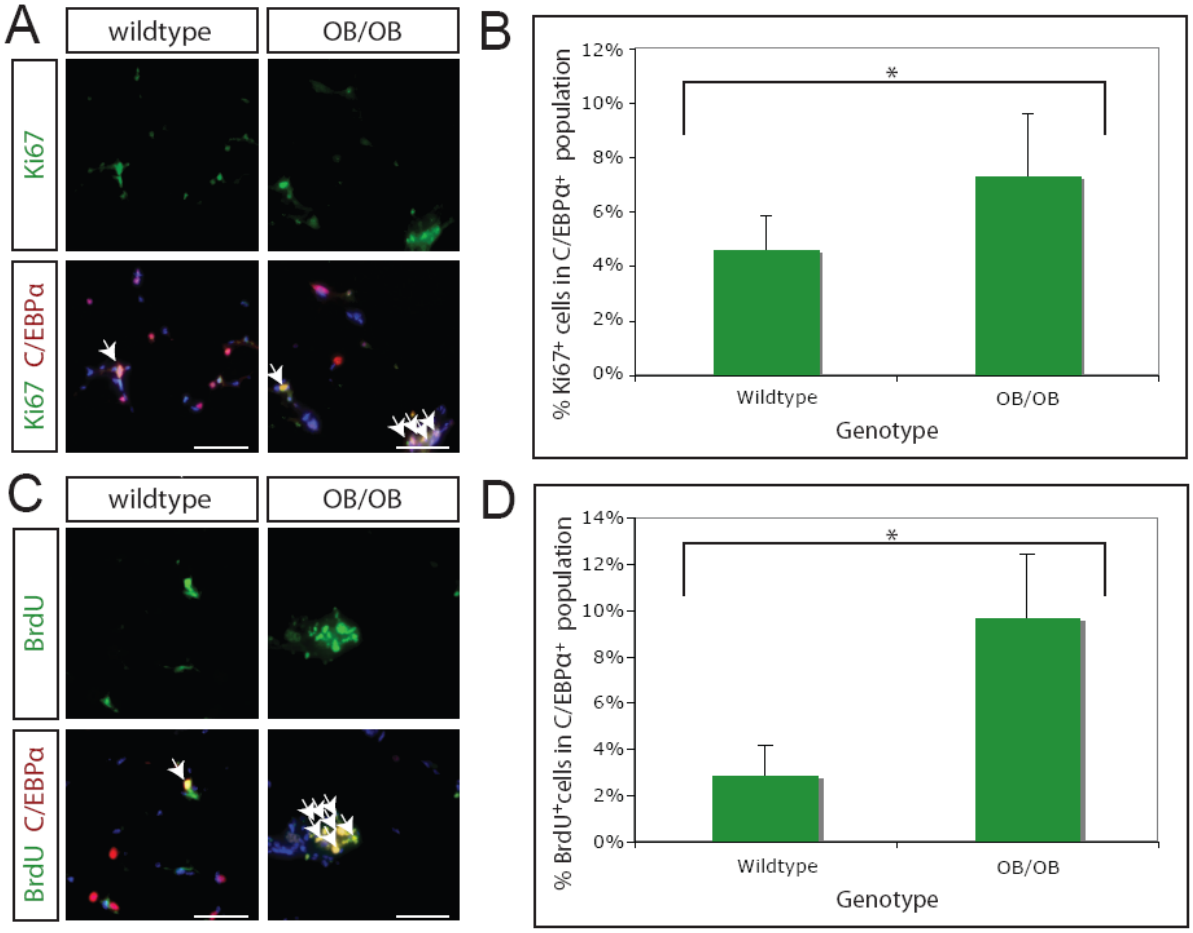

Figure 5.1. Increased fat cells replication in 14 weeks old OB/OB mice.

A. The rate of adipose tissue replication was assayed by co-staining for the cell cycle protein Ki67 (green) and the marker C/EBPα (red) in 14-week-old wild-type and OB/OB mice. Representative images of wild-type and OB/OB adipose tissue are shown. White arrows indicate cells co-staining for Ki67 and C/EBPα. DAPI stained nuclei are blue. Magnification, 200x; scale bars 100μm.

B. The percentage of C/EBPα-expressing cells in the growth cycle was quantified for 14-week old C57/BL6 wild-type (n=9) and OB/OB (n=6) mice. There is a statistically significant increase in the rate of adipose tissue replication ($p<0.05$) from 4.6% to 7.3%, in wild-type and OB/OB mice, respectively. Error bars indicate standard deviation.

C. The rate of adipose cells replication was assayed by co-staining for BrdU incorporation (green) and the adipose marker C/EBPα (red) in 14-week-old wild-type and OB/OB mice. Representative images of wild-type and OB/OB adipose tissue are shown. White arrows indicate cells co-staining for BrdU and C/EBPα. DAPI stained nuclei are blue. Magnification, 200x; scale bars 100μm.

D. The percentage of C/EBPα-expressing cells having incorporated BrdU following 5 days of BrdU injection was quantified for 14-week old C57/BL6 wild-type (n=5) and OB/OB (n=6) mice. There is a statistically significant increase in the rate of adipose tissue replication ($p<0.05$) from 2.9% to 9.7%, in wild-type and OB/OB mice, respectively. Error bars indicate standard deviation.

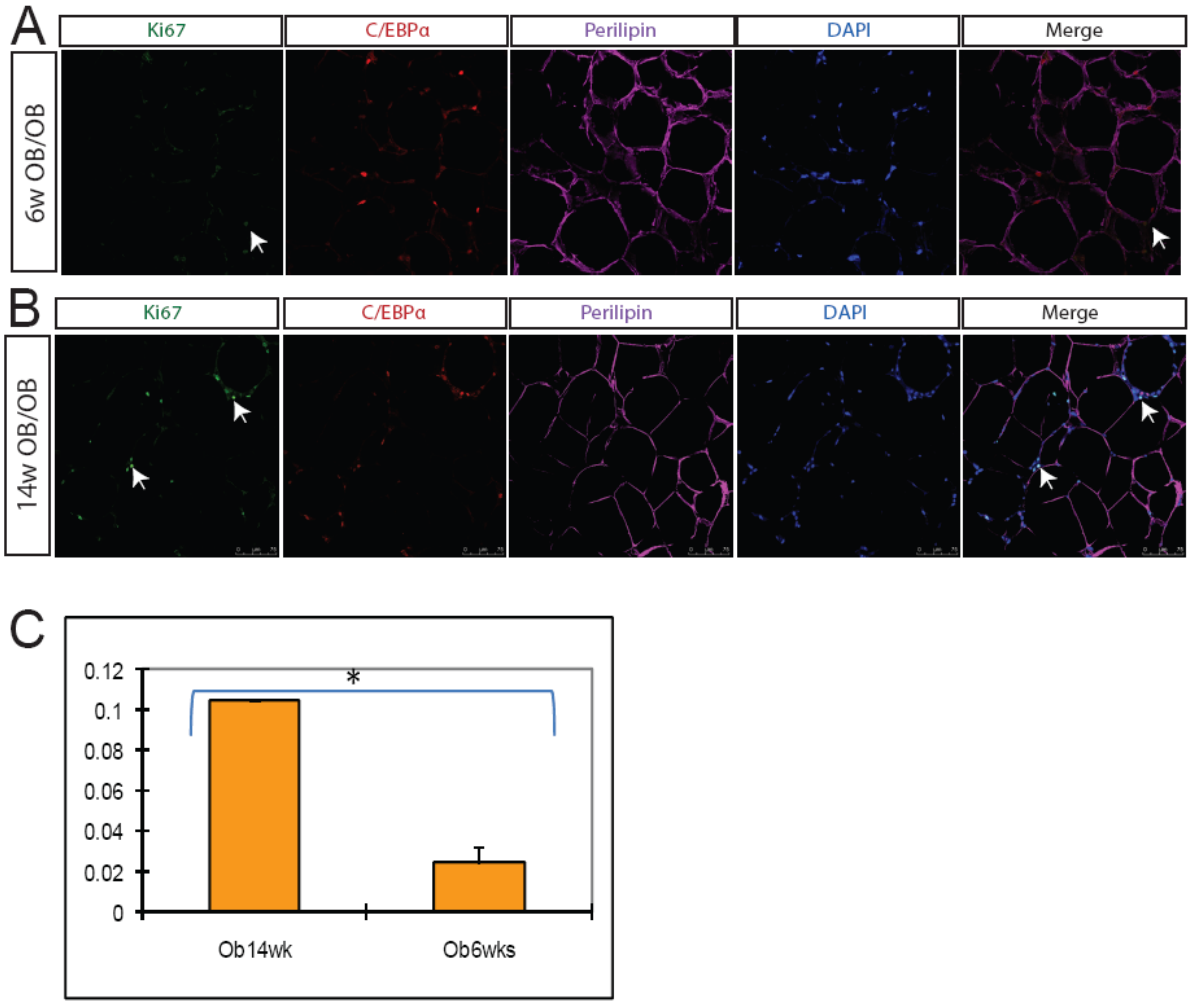

Figure 5.2. Adipose tissue in 6 weeks old OB/OB mice don't show significant increased replication.

A. By confocal microscopy, in 6-week old OB/OB mice, Ki67 (green) overlaps with (C/EBPα (red) *in situ*. Perilipin is stained in red. White arrow indicates double positive cell. DAPI stained nuclei are blue. Magnification, 200x; scale bars 50μm.

B. By confocal microscopy, in 14-week old OB/OB mice, Ki67 (green) overlaps with (C/EBPα (red) *in situ*. Perilipin is stained in red. White arrow indicates double positive cell. DAPI stained nuclei are blue. Magnification, 200x; scale bars 50μm.

C. The percentage of C/EBPα -expressing cells in the growth cycle within Perilipin population, was quantified for 6-week old OB/OB (n=3) and OB/OB (n=1) mice. There is a statistically significant increase in the rate of adipose cells replication ($p<0.05$) from 2.4% to 10.4%, respectively. Error bars indicate standard deviation.

Obesity is commonly considered to be a disorder of energy imbalance in the organism. At the cellular level, however, the development of obesity has been attributed to both an increase in fat cell size, fat cell differentiation and increased number of fat cells [Bjorntorp, P. et al. 1995, Sjostrom, L. & Bjorntorp, P. 1974, Faust et al, 1978, Wise 1975]. Our results support these notions, showing increase in adipose tissue replication associated with obesity in 14 weeks old OB/OB mice.

Interestingly, although we counted a small number of nuclei, our preliminary data didn't show an increase in replication in young (6 weeks old) OB/OB mice. One possible interpretation would be that fat cells increase first in size up to a point that triggers increased progenitor replication and differentiation. Additionally, given the results presented above, we cannot rule out the possibility that mature fat cells may be able to divide. Consequently, increased replication of mature adipocytes may at least partially contribute to the results seen in OB/OB mice. Finally, it has been previously reported that adipose tissue in obese mice is characterized by a substantial infiltration of macrophages which could contribute to the increased number of Ki67 positive cell. It would be important to calculate the number of Mac1 Ki67 positive cells in these mice.

Diet Induced Obesity (DIO) mice do not show increased adipose tissue replication

We next sought to determine whether adipose tissue replication changes in diet induced obesity (DIO) mice. DIO mice are C57/BL6 mice fed with high fat caloric diet since the day of weaning. To ask whether adipose tissue replication is altered in DIO mice, we determined the percentage of cells in cell cycle in 14-week-old DIO (n=3) mice. Ki67, and C/EBPα co-expressing cells were counted within the Perilipin population and found to constitute 3.6% of the total number of cells in the adipose tissue, while the proportion of cells in adipose tissue expressing Ki67 was 4.03% (Table 7). Our preliminary experiment didn't show statistically significant difference in adipose tissue replication in 14weeks old DIO mice compared to the wild type animals. ($p>0.05$)

Joe and colleagues (Joe et al. 2009) showed that in mice fed with high-fat diet, visceral adipose tissue was expanding predominantly by adipocyte hypertrophy, whereas subcutaneous adipose tissue was characterized by adipocyte hyperplasia. Furthermore, *in vivo* experiments where cells in S-phase were labeled with bromodeoxyuridine (BrdU) revealed a proliferative response to high-fat diet in subcutaneous adipose tissue progenitors, but not in visceral adipose depots. Our experiments performed on epididymal adipose tissue are compatible with this report.

Several reports have described significant differences between visceral and subcutaneous adipose depots in affecting metabolic health (Rajala MW. 2003, Livingston 1972). It is thought that hypertrophy-induced adipocyte dysfunction leads to alterations in glucose homeostasis and changes in adipocyte secretion resulting in metabolic disease, whereas subcutaneous adipocytes are suggested to be not contribute to the pathogenesis of such conditions. Joe and colleagues

propose that the ability of a depot to expand by hyperplasia determines whether a depot will remain functional and less associated to higher risk of disease.

These studies are interesting and provocative and may have important implications for the fields of adipose tissue homeostasis, turnover and metabolic disease, warranting further research in this field.

Murine adipose tissue replication is diminished during the second half of pregnancy.

It has been reported that in humans, the maternal metabolism undergoes substantial modifications in order to support the nutritional needs of the fetus during pregnancy. (Cetin I. et al 2005, Cetin I. et al 2009). During the first trimester, hyperphagia causes an increase in the mother's body weight, leading to lipid storing in adipose tissue (Prentice and Golberg 2000, Herrera 2002, Cetin et al. 2009). On the other hand, in the third trimester of pregnancy, the lipid metabolism of the mother is modified by switching to a catabolic condition in order to support the increased tissue growth of the fetus and its enhanced nutritional exigency (Felig et al 1970).

We sought to determine whether the rate of adipose cells replication is altered during mice pregnancy. A group of 30 C57/BL6 females 6-weeks-old mice were mated, 15 animals were analyzed for the first half of the pregnancy, while the remaining mice were studied for the second half of their pregnancy. For the first group of mice, the day the vaginal plug was detected (embryonic day 0, E0), the animals received daily BrdU injection. 4 animals were sacrificed the day after the first injection, (E0.5 n=4), and the remaining animals were sacrificed every two or three days after vaginal plug detection: (E2.5; n=4), (E4.5; n=2), (E6.5; n=2), (E9.5; n=3). (Figure 7.1A). The percentage of BrdU and Perilipin co-expressing cells were counted and fat cell incorporation of

BrdU was determined to be 0.9% per day (Figure 7.1A.), compared to 0.6% in the wild type mice. ($p<0.05$) (Table 10).

To analyze the second part of the pregnancy, we started the daily BrdU injection one week after the vaginal plug was detected and the mice were sacrificed every two or three days: (E8.5; n=3), (E10.5; n=3), (E14.5; n=3) (Figure 7.1B). The percentage of BrdU and Perilipin co-expressing cells was determined to be 2% at any time point analyzed. These data showed a decrease of BrdU incorporation over the last part of the pregnancy. (Figure 7.1).

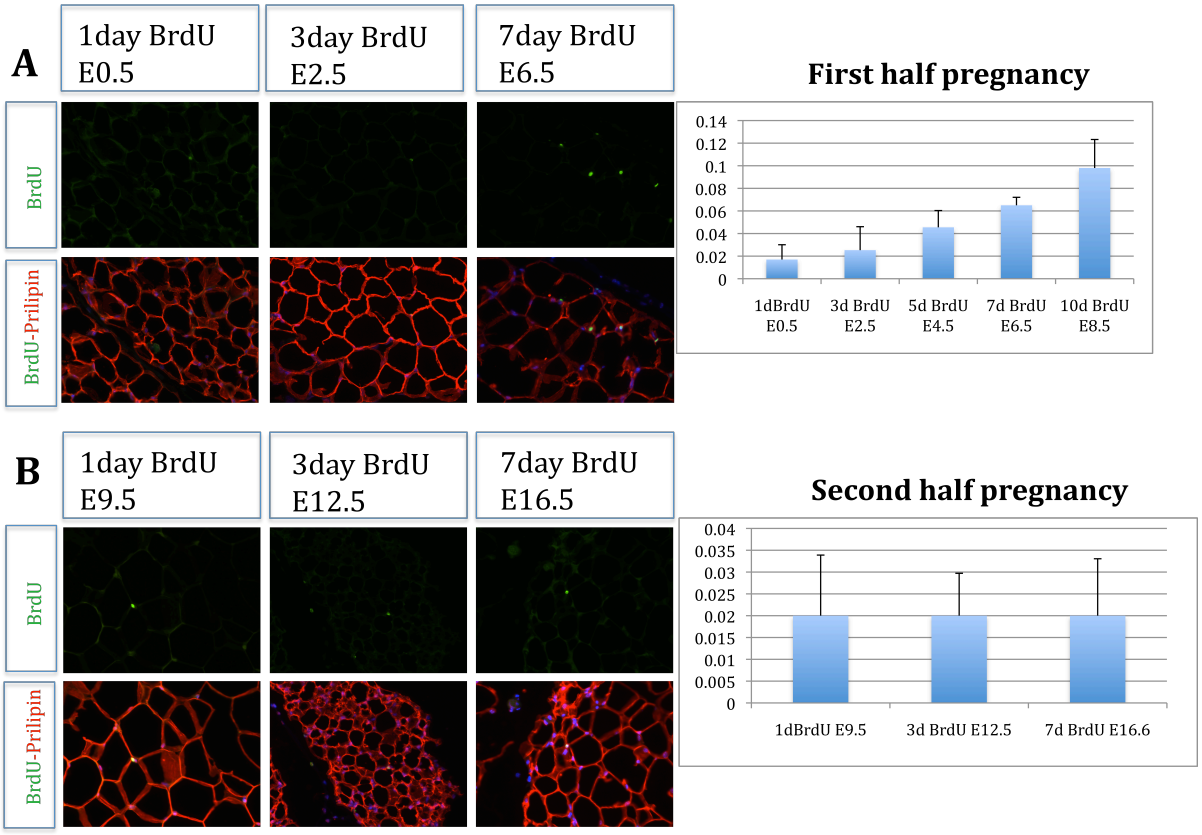

Figure 7.1. Adipose tissue replication assayed by immunohistochemistry for BrdU incorporation during pregnancy The rate of replication was assayed by BrdU incorporation in 6-week old C57BL/6 pregnant mice. A. First half of pregnancy: After the vaginal plug was detected, BrdU was injected intraperitoneally daily for 1 (n=7), 3 (n=7), 5 (n=6), 7 (n=9) or 10 (n=7) continuous days. 0.9% of fat cells incorporated BrdU per day. Perilipin staining is shown in red, BrdU staining in green, DAPI stained nuclei are blue. Magnification, 200x;
B. Second half of pregnancy: After 8 days the vaginal plug was detected, BrdU was injected intraperitoneally daily for 1 (n=2), 3 (n=3), 7 (n=3) continuous days. After 7 days of injection 2% of fat cells incorporated BrdU . Perilipin staining is shown in red, BrdU staining in green, DAPI stained nuclei are blue. Magnification, 200x;

We next determined the percentage of cells within the adipose tissue in the cell cycle during pregnancy. We counted Ki67 postive cells within Perilipin expressing cells and determined them to be 3.5% of the total population of cells in adipose tissue, compared to 1.8% in wild type mice. (Figure 7.2 Table 11)

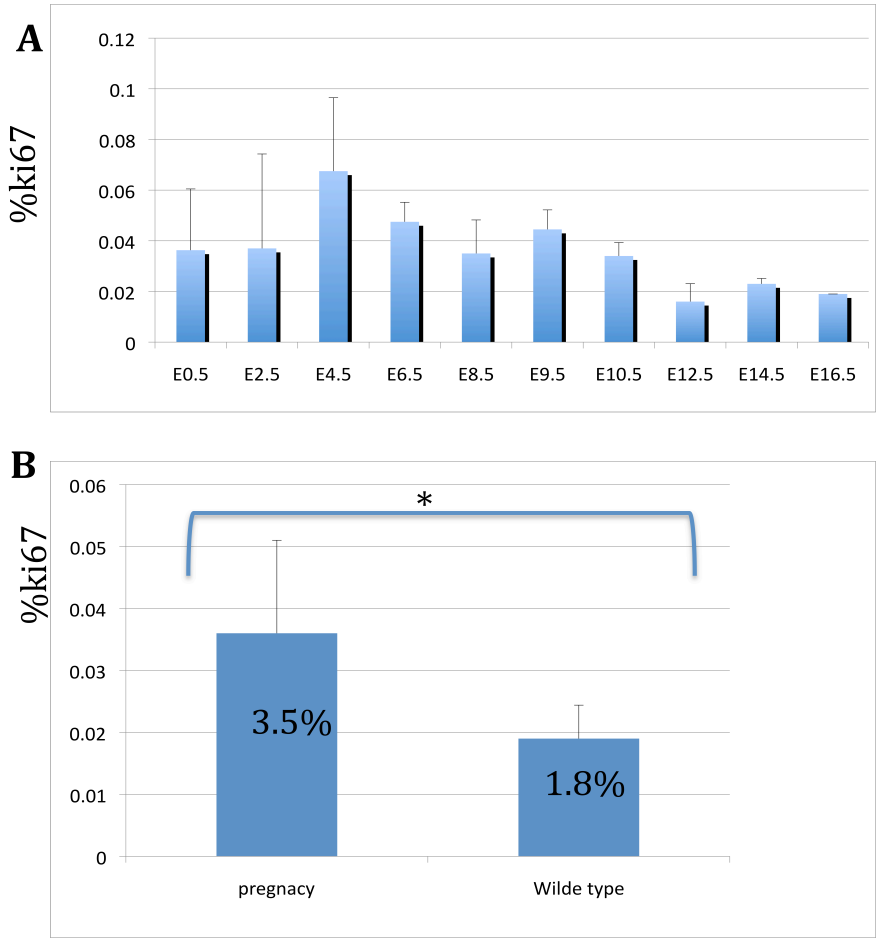

Figure 7.2. Adipose cells replication assayed with the cell cycle protein Ki67 during pregnancy.
A.The percentage of cells within Perilipin cells population in the growth cycle was quantitated in 6 week-old C57/BL6 pregnant mice over the course of the pregnancy. Approximately 3.5% of cells are Ki67 positive within the Perilipin positive population at any time. Error bars indicate standard deviation B.We found statistical significant difference between 6 weeks old pregnant mice and wilde type.

As mentioned previously, it is important to note that cells that at the time of our analysis were Perilipin, BrdU double positive could have incorporated BrdU when they were preadipocytes, and thereafter differentiated in mature fat cells. Furthermore, another previously discussed important issue is the technical difficulties in definitively distinguishing mature fat cells from other cells present in adipose tissue. Due to these technical issues, we cannot with 100% certainty exclude that the Perilipin, Ki67 positive population does not include endothelial cells, macrophages and preadipocytes.

Although we cannot with 100% certainty identify replicating adipcytes, our experiments suggest an increase in adipose tissue replication over the first half of pregnancy, supporting the notion that the first period of gestation is characterized by an anabolic state in which the mother increases her adipose tissue deposition in order to support the development and growth of the fetus.
It is believed that during the second part of pregnancy, fat metabolism in the mother is switched to a catabolic condition, thereby providing sufficient nutrients to the fetus as it increases its nutritional demands. Our BrdU incorporation experiments, where a decrease of adipose tissue replication during the last part of the pregnancy was detected, are compatible with this model.

No statistically significant increase in adipose tissue replication following lipectomy

Several studies have used surgical excision of a specimen from a renewable organ to investigate whether a burst in proliferation occurs (Bucher N. et al 1965, Faust I. et al 1977). It has been reported that epididymal fat pads in young rats and mice failed to regenerate after surgical excision, whereas regeneration of subcutaneous adipose tissue in rats lipectomized at 3 weeks of age and fed a high fat diet beginning at 12 weeks of age was observed to be complete after 7 months after surgery, in contrast to the incomplete regeneration achieved on adipose depots of rats fed normal chow diet (Faust I. et al, 1976, Liebelt et al. 1965, Kral. et al 1976).

We sought to investigate whether the rate of adipose tissue replication changes after lipectomy. A group of 10 C57/BL6 6 months old mice underwent epididymal lipectomy after they were anesthetized by intraperitoneal injection of Avertin. A small incision was performed between the second and third nipple in the mouse's lower stomach, on both left and right side, cutting through the skin and the muscle layer. In 6 animals, on the right side of the mouse, a specimen of epididymal fat (60%) was removed, weighed, fixed, and embedded in OCT for immunoistochemistry analysis. In the remaining 4 sham-operated animals we performed the surgery without removal of any adipose depots. The wounds were stitched using silk and needle. Mice were given daily injections of BrdU from the day of the surgery and 7 days onwards. On the 8^{th} day the animals were sacrificed. Sections of adipose tissue were stained with Perilipin and BrdU and the percentage of BrdU positive cells were

counted within the Perilipin positive cell population (Figure 8). In the lipectomized mice, we found that in the adipose tissue where fat was removed, 10.9% of adipose cells incorporated BrdU, while in the adipose depots where fat was not removed, 4.4% of the cells incorporated BrdU. As shown in the chart, there was no statistically significant difference in replication between the two fat depots (p=0.07). We then counted the percentage of adipose cells that incorporated BrdU in sections of adipose tissue from the sham group who underwent surgery without fat removal. Here, the number of Perilipin, BrdU double positive cells was found to be 4% in both the operated and the non-operated side (Figure 8, Table 12).

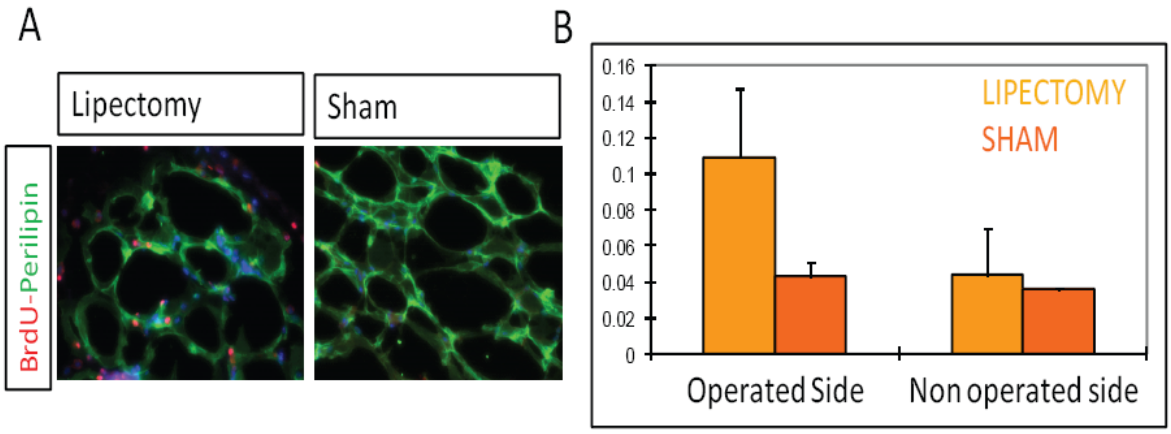

Figure 8. Adipose cells replication assayed by immunohistochemistry for BrdU incorporation after lipectomy. A the rate of fat cells replication was assayed by BrdU incorporation in 6 months old C57BL/6 mice after lipectomy or sham surgery. The day of surgery BrdU was injected intraperitoneally daily for7 (lipectomy n=9; sham n=4)continuous days. Perilipin staining is shown in green, BrdU staining in green, DAPI stained nuclei are blue. Magnification, 200x. B.Mice who underwent lipectomy: The percentage of BrdU incorporation was found to be 10% in adipose depots which underwent lipectomy and 4.4% in the non operated side (p-value=0.07). Sham: the percentage of BrdU incorporation was found to be 4.2% in the adipose tissue of the operated side and 3.6% in the adipose depots which didn't go under surgery.

Thus, we failed to detect a statistically significant increase in adipose tissue proliferation in mice 1 week after a lipectomy procedure. The discrete increase in the number of proliferative cells we observed (10% compared to 4.4% of BrdU incorporation) could be macrophages which infiltrated the tissue due to an inflammatory response elicited by the surgical procedure. Alternatively, in response to the surgical procedure, adipose cells or preadipocytes may increase their rate of proliferation in order to regenerate the loss of fat tissue. It would be interesting to investigate whether the proliferation of adipose tissue changes after lipectomy in mice fed a high fat diet and in OB/OB mice. Moreover it would be interesting to determine whether the rate of proliferation changes after longer periods of time following the lipectomy procedure. Finally, we focused our analysis on epididymal fat depots; since several studies suggested a regeneration in subcutaneous versus epididymal adipose tissue, it may be of interest to investigate the rate of replication in lipectomized subcutaneous adipose tissue and compare the results to the data presented above as well as with other previous reports.

Evidence for replication of human adipose cells.

To investigate the replication potential of the human adipose tissue, we assessed a known marker of cell division within a population of human adipose cells *in vivo*. Using confocal microscopy of stained sections of human adipose tissue, we determined the percentage of C/EBPα positive cells co-epressing Ki67 within the Perilipin population (Figure 8; Table 13). We found that 1.5% of total adipose tissue is Ki67-positive, and that 0.7% of cells in adipose tissue are positive for Ki67, C/EBPα within Perilipin expressing cells. (Figure 8B; Table 13). As previously discussed for mouse adipose tissue, the Ki67-C/EBPα expressing cells within the Perilipin population may be preadipocytes or macrophages infiltrated in the tissue. It would be interesting to count the percentage of dividing macrophages and vascular cells in this tissue to determine whether they constitute all Ki67-positive cells in this population.

Overall, our experiments indicate replication in human adipose tissue, and quantify the total number of cells in growth phase.

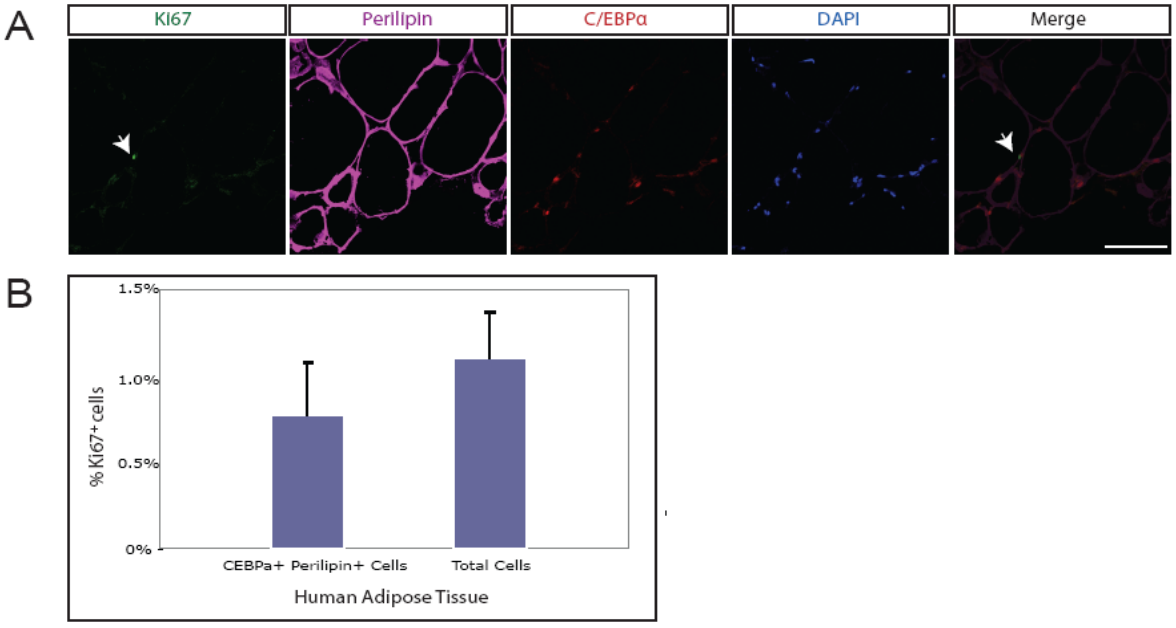

Figure 9. Human adipose tissue replication assayed with the cell cycle protein Ki67.
A. Ki67 (green) overlaps with C/EBPα (red) in situ. Perilipin in purple. White arrow indicates double positive nuclei. DAPI stained nuclei are blue. Magnification, 200x; scale bars 50μm.
B. The percentage of adipose cells in the growth cycle was quantitated in human adipose tissue. 0.7% of cells in adipose tissue are positive for Ki67, C/EBPα within Perilipin population at any time, while 1.5% of total adipose tissue is Ki67-positive. Error bars indicate standard deviation.