CLIC3 controls recycling of late endosomal MT1-MMP and dictates invasion and metastasis in breast cancer

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Word count 3,236

Key words:
CLIC3, DCIS, invasion, MT1-MMP, breast cancer, ER-negative, recycling, late endosome.

Short title:
CLIC3, MT1-MMP trafficking and breast cancer
SUMMARY (180 words)

Chloride intracellular channel 3 (CLIC3) drives invasiveness of pancreatic and ovarian cancer by acting in concert with Rab25 to regulate recycling of $\alpha_5\beta_1$ from late endosomes to the plasma membrane. Here we show that in two estrogen receptor (ER)-negative breast cancer cell lines CLIC3 has little influence on integrin recycling, but controls trafficking of the pro-invasive matrix metalloprotease, MT1-MMP. In MDA-MB-231 cells MT1-MMP and CLIC3 are localised primarily to late endosomal / lysosomal compartments located above the plane of adhesion and near the nucleus. MT1-MMP is transferred from these late endosomes to sites of cell-matrix adhesion in a CLIC3-dependent fashion. Correspondingly, CLIC3 knockdown opposes MT1-MMP-dependent invasive processes. These include the disruption of the basement membrane as acini formed from MCF10DCIS.com cells acquire invasive characteristics in 3D culture, and the invasion of MDA-MB-231 cells into Matrigel or organotypic plugs of type I collagen. Consistent with this, expression of CLIC3 predicts poor prognosis in ER-negative breast cancer. The identification of MT1-MMP as a cargo of a CLIC3-regulated pathway that drives invasion highlights the importance of late endosomal sorting and trafficking in breast cancer.
Introduction

Invasion through the basement membrane and surrounding stroma is necessary for breast cancer to progress beyond the ductal carcinoma in situ (DCIS) stage. Matrix metalloproteases, in particular MT1-MMP, are required for carcinoma cells, to migrate across basement membranes (Hotary et al., 2006), through fibrillar type I collagen (Li et al., 2008; Sabeh et al., 2004), and for invasion and metastasis in vivo (Perentes et al., 2011; Sabeh et al., 2004; Szabova et al., 2008). Consistently, MT1-MMP is upregulated with increasing grade and stage of breast cancer (Ueno et al., 1997), and its expression is associated with poorer survival (McGowan and Duffy, 2008; Tetu et al., 2006). It is increasingly apparent that endocytosis and recycling of MT1-MMP provide an important level of regulation (Frittoli et al., 2011; Poincloux et al., 2009; Uekita et al., 2001; Williams and Coppolino, 2011).

We have recently shown how CLIC3 and Rab25 can cooperate to drive endocytic trafficking of active β1-integrin from the late endosome / lysosome to the plasma membrane at the rear of the cell, promoting disassembly of cell-matrix adhesion and facilitating migration (Dozynkiewicz et al., 2012). We highlighted the reduced survival following resection of operable pancreatic cancers where levels of CLIC3 were high and showed that the poorest outcomes occurred where both Rab25 and CLIC3 were elevated. Here we report that CLIC3-dependent recycling can operate independently of Rab25 to facilitate the polarised delivery of MT1-MMP to the substratum in ER-negative MDA-MB-231 cells and show that expression of CLIC3 is associated with reduced survival in ER-negative breast cancer.
Results and Discussion

**CLIC3 expression is increased in breast cancer and is associated with poor outcome.**

To determine CLIC3 expression in breast cancer we analysed a tissue microarray containing primary breast carcinomas and paired adjacent normal tissue. CLIC3 staining was typically low in normal ductal epithelium with significantly higher expression in matched tumour tissue (Fig. 1A, B; Fig. S1A, B). Consistently, the Oncomine database (Rhodes et al., 2004) identified higher levels of CLIC3 mRNA in invasive carcinomas than normal breast tissue in both the TCGA (fold increase = 2.12, P = 3.18E-18) and Gluck (fold increase 1.60, P = 1.83E-5) (Gluck et al., 2012) datasets (Fig. S1C, D). Next we investigated the relationship between CLIC3 and survival in a cohort of 141 women with ER-negative early breast cancer (Fig. 1C, Table S1). High CLIC3 expression was associated with poorer cancer-specific survival (p=0.026, log rank test) (Fig. 1C). Although CLIC3 expression was higher in patients with HER2-positive disease the effect of CLIC3 on survival was most evident in the HER2-negative (“triple-negative”) subset (Fig. S1E). To address whether the negative prognostic significance of CLIC3 expression could be observed in other cohorts, we analysed gene expression data from 198 node-negative, systemically untreated patients (Desmedt et al., 2007). High CLIC3 mRNA was associated with worse overall survival in the overall cohort (P=0.039, log rank test) (Fig. 1D) and in the subset of ER-negative (n=64; P=0.005, log rank test) (Fig. 1E), but not ER-positive (Fig. 1F) patients. These data show that CLIC3 is overexpressed in some primary breast cancers and that high levels of CLIC3 are associated with a greater risk of death.

**CLIC3 is involved in matrix degradation and invasion but not proliferation.**

To investigate how CLIC3 might contribute to aggressiveness in breast cancer we silenced its expression in MDA-MB-231 cells, observing no alteration of growth in vitro (Fig. S2A, B). We hypothesised that CLIC3 might promote an invasive phenotype similar to that observed in Rab25-expressing ovarian tumours (Dozynkiewicz et al., 2012). However, although Rab25 has been identified as the driver of the 1q22 amplicon which promotes ovarian cancer aggressiveness (Cheng et al., 2004), it appears to inhibit progression in several other tumour types (Amornphimoltham et al., 2013; Nam et al., 2010; Tong et al., 2012). In ER-negative breast cancer loss of Rab25 is common (Cheng et al., 2006). MDA-MB-231 cells do not express Rab25 and forced re-expression reduces their invasiveness (Cheng et al., 2010).
Consistent with these latter observations we could not detect any association between Rab25 expression and poor outcome in ER-negative breast cancer, nor any clear indications of collaboration between Rab25 and CLIC3 (Fig. S1F). This led us to postulate the presence of a distinct CLIC3-dependent but Rab25-independent pro-invasive pathway in the context of some ER-negative breast carcinomas. Consistent with this hypothesis, two CLIC3 siRNAs reduced invasion through fibronectin-supplemented Matrigel (Fig. 2A) with invasion rescued by expression of siRNA-resistant CLIC3 (Fig. S2C). Silencing CLIC3 expression also reduced invasion into organotypic collagen plugs which had been preconditioned with dermal fibroblasts (Fig. 2B).

A key event in progression from DCIS to invasive carcinoma is proteolytic disruption of the basement membrane. To model this event in 3D culture, we employed ER-negative MCF10DCIS.com mammary cells that are derived from the ‘normal’ MCF10A cell line and form well-defined comedo-like DCIS structures when injected as xenografts. However, with time these lesions spontaneously progress to invasive carcinoma characterised by disruption of their surrounding basement membrane, and the development of invasive outgrowths (Behbod et al., 2009; Hu et al., 2008; Miller et al., 2000). Elements of this progression are recapitulated in 3D culture (Jedeszko et al., 2009; So et al., 2013). Indeed, when cultured in Matrigel for 2-4 days, MCF10DCIS.com cells formed non-invasive comedo structures bounded by a basement membrane (as determined by immunofluorescence staining for laminin-5, α6 and β4 integrin and collagen (not shown). However, quantitative morphological analysis indicated that following 6 days of culture these comedo-like structures began to lose their sphericity (Fig. 2C), and that this was associated with appearance of substantial breaches of the basement membrane through which cells invaded into the surrounding Matrigel (Fig. 2C). Silencing of CLIC3 significantly opposed this spontaneous disruption of the basement membrane and loss of acinar sphericity (Fig. 2C; Fig. S2D). Taken together these data implicate CLIC3 in disruption of the basement membrane and the invasive behaviour that accompanies DCIS to invasive carcinoma transition.

**CLIC3 localises to a late endosomal / lysosomal compartment**

Most CLIC3 in MDA-MB-231 cells was present at vesicles (Fig. 3A, B). To determine which compartment(s) this represented, we coexpressed mCherry-CLIC3 with markers of early and recycling endosomes (EEA1, Rab4, Rab11a), late endosomes/lysosomes (Rab7, LAMP1 &
Lysotracker Green) and the Golgi (pAcGFP1-Golgi). The majority of CLIC3 was present in vesicles that contained Rab7, LAMP1 or Lysotracker (Fig. 3A; Fig. S3A). Thus, in MDA-MB-231 cells, CLIC3 is predominantly localised to the late endosomal/lysosomal system consistent with previous studies in A2780 ovarian carcinoma cells (Dozynkiewicz et al., 2012).

**CLIC3 is required for recycling of internalised MT1-MMP**

The involvement of CLIC3, a late endosomal protein, in disruption of the basement membrane - an event that is known to require proteolysis of basement membrane components - led us to consider MT1-MMP, a transmembrane matrix metalloprotease that traffics through late endosomes (Poincloux et al., 2009). Indeed, siRNA of MT1-MMP opposed invasion of MDA-MB-231 into Matrigel (Fig. S4A). Furthermore, the MT1-MMP inhibitor, GM6001, opposed spontaneous loss of comedo sphericity (Fig. S4B) and basement membrane disruption (Fig. S4C), indicating involvement of MT1-MMP in these processes. MT1-MMP undergoes endo-exocytic cycling, and previous studies have implicated recycling pathways involving late as well as early endosomes and the trans-Golgi network in returning internalised MT1-MMP to the plasma membrane (Bravo-Cordero et al., 2007; Remacle et al., 2003; Remacle et al., 2005; Wang et al., 2004; Williams and Coppolino, 2011). In MDA-MB-231 cells, MT1-MMP is predominantly localised to the late endosomes/lysosomes from where it is recycled to the plasma membrane (Monteiro et al., 2013; Steffen et al., 2008; Yu et al., 2012). Indeed, we found that $21 \pm 1.27\%$ (mean\(\pm\)SEM, n=26 cells) of endogenous MT1-MMP resided in vesicles that were positive for endogenous CLIC3 (Fig. 3B), and live cell imaging of mCherry-CLIC3 and GFP-MT1-MMP revealed a subset of vesicles in which these proteins were colocalised and moved together for extended periods (Fig. 3C, Fig. S3B, video S1).

We speculated that CLIC3 might have a role in delivery of internalised functional MT1-MMP from late endosomes back to the plasma membrane. In control MDA-MB-231 and MCF10DCIS.com cells, recycling of MT1-MMP proceeded with bi-phasic kinetics; an initial phase of recycling which occurred within 5 min, followed by a period of slower recycling (Fig. 3D). Whilst total levels of MT1-MMP were not altered by CLIC3 knockdown (not shown), the rate at which internalised MT1-MMP was returned to the plasma membrane was reduced, particularly in the initial rapid phase of recycling (Fig. 3D). A more modest effect was observed for $\alpha 5\beta 1$-integrin (data not shown). Recycling of MT1-MMP was rescued by
expression of siRNA-resistant CLIC3 (Fig. 3E). It is important to note that the rapid kinetics observed do not necessarily entail “short loop” Rab4-dependent recycling of MT1-MMP from the early endosome. Indeed, we observed that silencing of Rab27A and Rab27B (Fig. S2E), which are established regulators of exocytic trafficking from late endosomal (Ostrowski et al., 2010) and secretory lysosomal compartments (Menasche et al., 2000), as well as related organelles such as melanosomes (Bahadoran et al., 2001), was sufficient to inhibit rapid recycling of MT1-MMP in a manner similar to that observed with CLIC3 silencing (Fig. 3F).

**CLIC3 is required for delivery of MT1-MMP vesicles to the substratum**

Z-sections taken through the cell body indicated that the amount of vesicular (endogenous and mCherry-tagged) MT1-MMP near the ventral surface of the cell was reduced by CLIC3 knockdown (Fig. 4A, B; Fig. S3C). We used TIRF video-microscopy to quantify appearance of MT1-MMP containing vesicles very close (<200 nm) to the ventral plasma membrane. Silencing of CLIC3 reduced the number and size of MT1-MMP containing vesicles that appeared in the TIRF field (Fig. 4C), and correspondingly increased the amount of MT1-MMP retained within perinuclear vesicles above the plane of adhesion (Fig. 4B). Expression of siRNA-resistant CLIC3 rescued delivery of MT1-MMP to the TIRF field (Fig. 4D). CLIC3 knockdown did not reduce the amount of LAMP1 vesicles in the TIRF field indicating that CLIC3 was not required for delivery of late endosomes and/or their docking with the plasma membrane (Fig. 4E). These observations suggest that CLIC3 may act in the perinuclear region to promote sorting of MT1-MMP into late endosomes destined for the plasma membrane, rather than being necessary to achieve delivery of late endosomes in general.

Collectively, our data identify CLIC3 as a regulator of the sorting and targeting of MT1-MMP containing late endosomes to the ventral plasma membrane, and indicate that CLIC3-dependent delivery of MT1-MMP promotes MT1-MMP-dependent invasiveness. Our previous work identified a collaborative role for CLIC3 and Rab25 to drive α5β1 recycling in ovarian and pancreatic cancer. However, the present study conducted in ER-negative breast cancer cells, including MDA-MB-231 cells that do not express Rab25, reports minor reductions in integrin recycling following CLIC3 knockdown, whereas trafficking of MT1-MMP is strongly CLIC3-dependent. It is perhaps not surprising that non-integrin cargoes of the CLIC3-dependent pathway should not be influenced by Rab25 expression. Indeed, Rab25
associates directly with $\alpha_5\beta_1$ integrin, and in the absence of this interaction this integrin cannot efficiently be targeted to late endosomes (Dozynkiewicz et al., 2012). MT1-MMP, however, can clearly reach CLIC3-positive late endosomes without Rab25. Moreover, invading MDA-MB-231 cells traffic MT1-MMP to invadopodia located towards the leading edge of the cell (Packard et al., 2009; Wolf et al., 2007; Yu et al., 2012), whereas CLIC3-dependent pathways transport $\alpha_5\beta_1$ integrin to the cell rear in Rab25 expressing cells. Therefore we propose that CLIC3 is involved in late endosomal trafficking to drive invasion in a broad range of tumour types, but that the particular pro-invasive cargo (active integrin versus MT1-MMP) and the site to which recycling is targeted (cell rear versus invadopodia) will depend on the cellular context and expression of GTPases such as Rab25.
Materials and Methods

TMA and survival analysis

Generation of the breast TMA, CLIC3 immunohistochemistry and use of the weighted histoscore method are described previously (Dozynkiewicz et al., 2012; Ohotski et al., 2012). Protein competition and matched normal and tumor tissue studies utilized TMAs T089 and BR804a respectively (US Biomax). Details of antibodies are in Table S2.

Cell culture and nucleofection

MDA-MB-231 cells were cultured and transfected as described previously (Yu et al., 2012). MCF10DCIS.com cells were cultured in Advanced DMEM/F12, L-Glutamine and 5% Horse Serum and nucleofected using Amaxa kit T / Program T-020. MDA-MB-231 cells expressing the siRNA-resistant pcDNA3-FLAG-CLIC3 (Dozynkiewicz et al., 2012) or pcDNA3 were generated by selection in G418 (800 μg / ml). siRNA sequences: CLIC3 #3: AGACAGACACGCUGCAGAU; #4: AGGACGUGCUGAAGGACUU; non-targeting smartPool; MT1-MMP smartpool; RAB27A and RAB27B smartpools (all Dharmacon). Cherry-CLIC3 and pcDNA3-Flag-CLIC3 constructs were described previously (Dozynkiewicz et al., 2012). mCherry-MT1-MMP was a gift from Philippe Chavrier (Institute Curie, Paris, France). pAcGFP1-Golgi from Clontech (Palo Alto, CA). GFP-Tensin 1 was a gift from Ken Yamada (NIH, Bethesda). GFP-α5 integrin was a gift from Donna Webb (Vanderbilt, Nashville, TN). EGFP-Rab4, EGFP-Rab11a, LAMP1-YFP, Rab7-GFP are as described previously.

For 3-D culture, 5x10^3 MCF10DCIS.com cells/well were plated on a thin layer of Matrigel (40 μl per well; or 5 μl for immunofluorescence studies) in an 8-well chamber slide as previously described (Debnath et al., 2003). Multiple phase contrast images at x10 magnification were captured from duplicate wells after 6 days of culture and circularity determined using ImageJ.

Invasion Assays

Invasion assays were performed as described previously (Hennigan et al., 1994; Timpson et al., 2011)

Recycling Assay

Recycling assays were performed as described previously (Roberts et al., 2001).
Immunofluorescence

Cells were seeded onto glass-bottomed 3 cm plates and imaged with a 64× objective of an inverted confocal microscope (Fluoview FV1000; Olympus) in 5% CO₂ at 37°C 48 hours after nucleofection of expression vectors or 30 min after addition of Lysotracker Green DND-26 (Molecular Probes). To image endogenous CLIC3 cells were trypsinised, fixed overnight in 10% neutral buffered formalin, resuspended in 2% agarose and embedded in wax. Proteins were then visualised by immunofluorescence of paraffin sections. To quantify colocalization the confocal images underwent two rounds of local contrast enhancement (image blurring, subtraction of the blurred image, and subsequent contrast enhancement) and threshold adjustment using ImageJ software. The number of yellow pixels was then expressed as a percentage of pixels in the red channel as previously described (Dozynkiewicz et al., 2012).
Acknowledgements
Work in the Norman Lab is funded by Cancer Research UK and the Breast Cancer Campaign. E. Rainero is funded by the West of Scotland Women’s Bowling Association. The breast cancer TMA was constructed by Dr Claire Orange and immunohistochemistry performed by Mr Colin Nixon.
References


Figure Legends

Figure 1. CLIC3 expression is associated with poor prognosis in early breast cancer. (A) Immunohistochemical staining of CLIC3 in a breast cancer TMA indicating predominantly negative (0), weak (1+), moderate (2+) and strong (3+) staining. (B) Box plot indicating CLIC3 histoscores in invasive carcinoma and paired adjacent normal breast tissue (n=40). P, Wilcoxon signed rank test. Examples of 2 cases with upregulated CLIC3 are shown. (C) Box plot illustrating stratification of patients with early breast cancer into high and low CLIC3 expressors based on histoscore (highest tertile versus lower two tertiles; H-score >123). Kaplan-Meier analysis indicates that patients with high CLIC3 protein expression have poorer breast cancer-specific survival. (D) Kaplan-Meier analysis performed in a cohort of 198 patients with resected early breast cancer (Desmedt; GSE7390) indicating poorer overall survival in patients with high CLIC3 expression (highest tertile versus lower two tertiles). P, log rank test. (E,F) Kaplan-Meier analysis of Desmedt data in subsets of patients with ER-negative (n=64; (E)) and ER-positive (n=134; (F)) disease. Scale bars, 100 μm.

Figure 2. CLIC3 promotes invasion. (A) MDA-MB-231 cells were nucleofected with non-targeting siRNA (si-con) or siRNAs targeting CLIC3 (si-CLIC3 #3 or #4) then allowed to invade into plugs of fibronectin-supplemented Matrigel. Invasion is quantified as the proportion of cells migrating further than 45 μm. The experiment was repeated using pools of MDA-MB-231 cells with stable expression of empty vector (MDA-MB-231-pcDNA3) or Flag-tagged siRNA-resistant CLIC3 (MDA-MB-231-Flag-CLIC3). Data are mean ± SEM from three independent experiments, performed in duplicate; P value, Mann-Whitney U test. (B) MDA-MB-231 cells were plated onto a collagen plug that had been pre-conditioned by primary human fibroblasts, allowed to invade for 6 days, then stained for cytokeratin. Scale bar, 100 μm. At least 4 individual plugs per siRNA duplex were generated in 2 independent experiments. Values are mean ± SEM; Mann-Whitney U test. (C) MCF10DCIS.com cells were plated on a thin layer of Matrigel and phase contrast micrographs captured after 6 days (upper panels). Individual acini were outlined and circularity determined using ImageJ with a value of 1 representing perfect circularity. Each data point represents a single acinus with data generated in three independent experiments, (si-con, n=121; si-CLIC3 #3, n= 242; si-CLIC3 #4, n=218); Bar, median; P value, Mann-Whitney U test. To visualise the basement membrane acini were stained for α6-integrin or laminin-5 (middle panels). The actin
cytoskeleton and nuclei were visualised with FITC-phalloidin and DAPI staining respectively (lower panels). Scale bars, 20 μm.

Figure 3. CLIC3 is localised to the late endosome / lysosome and regulates MT1-MMP recycling. (A) MDA-MB-231 cells expressing fluorescently tagged proteins or exposed to Lysotracker Green were imaged by live cell confocal microscopy. Colocalisation is expressed as a percentage of yellow versus red pixels for ≥ 30 cells from ≥ 3 experiments. Box, median and interquartile range; whiskers, range. Scale bar, 10 μm. Single channel images are presented in Fig. S3A. (B) Co-localisation of endogenous CLIC3 and MT1-MMP in fixed paraffin-embedded MDA-MB-231 cells. Examples of two separate cells, with differing focal planes are shown in the upper and lower panles respectively. Scale bar, 5μm (C) MDA-MB-231 cells expressing GFP-MT1-MMP and Cherry-CLIC3 were imaged by live cell confocal microscopy. Movies were acquired and stills shown from the time points indicated. Scale bar, 10 μm. Single channel images are presented in Fig. S3B. (D) MDA-MB-231 or MCF10DCIS.com cells were nucleofected with non-targeting siRNA (si-con) or siRNA targeting CLIC3 (si-CLIC3 #4). Recycling of MT1-MMP was determined as described in Roberts et al., (2001). Values are mean ± SEM from 3 independent experiments. (E) MT1-MMP recycling was determined following CLIC3 siRNA in MDA-MB-231 cells with stable expression of empty vector (pcDNA3) or Flag-tagged siRNA-resistant CLIC3 (Flag-CLIC3). (F) MDA-MB-231 cells were nucleofected with non-targeting siRNA or siRNAs targeting Rab27A and Rab27B (si-Rab27A+B) and recycling of MT1-MMP determined.

Figure 4. CLIC3 is required for polarised delivery of CLIC3 vesicles to the substratum. (A) MDA-MB-231 cells were nucleofected with non-targeting siRNA (si-con) or siRNA targeting CLIC3 (si-CLIC3 #4), fixed, and endogenous MT1-MMP detected with an anti-MT1-MMP antibody. Actin was stained with FITC-phalloidin and the nucleus with DAPI. (B) MDA-MB-231 cells were nucleofected with siRNA, cherry-MT1-MMP and GFP-tagged α5-integrin and images captured at both the ventral surface of the cell (plane of adhesion) and at the centre of the cell body (nuclear plane) with live cell confocal microscopy. ImageJ was used to quantify the amount of MT1-MMP that colocalised with α5-integrin; expressed as the percentage of yellow versus red pixels. Values are mean ± SEM from ≥ three experiments (n ≥ 30 cells). (C) MDA-MB-231 cells were nucleofected with siRNA, cherry-MT1-MMP and GFP-tagged Tensin-1 and imaged with live cell TIRF microscopy. Quantification of number of MT1-MMP vesicles entering TIRF field (left), total area occupied by MT1-MMP vesicles...
(centre) and average vesicle size (right) are shown. (D) MDA-MB-231 cells with stable expression of empty vector (pcDNA3) or Flag-tagged siRNA-resistant CLIC3 (Flag-CLIC3) were nucleofected with siRNA, cherry-MT1-MMP and GFP-tagged Tensin 1 and TIRF microscopy performed as above. (E) Live cell TIRF microscopy of MDA-MB-231 cells nucleofected with siRNA, LAMP1-Cherry and GFP-tagged Tensin 1. Values are mean ± SEM. P, Mann Whitney U test. Scale bars, 10 μm.
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A

Co-localisation

GFP-Rab4
Cherry-CLIC3

GFP-Rab11a
Cherry-CLIC3

GFP-Rab7
Cherry-CLIC3

GFP-Lamp1
Cherry-CLIC3

Lysootracker
Cherry-CLIC3

% colocalisation

CLIC3 colocalisation with:

B

CLIC3

MT1-MMP

MT1-MMP

CLIC3

zoom

zoom

zoom

C

Cherry-CLIC3

GFP-MT1-MMP

GFP-MT1-MMP

[Video S1]

D

MDA-MB-231

MCF10DCIS.com

E

F

si-con / pcDNA

si-CLIC3 #4 / pcDNA

si-con / Flag-CLIC3

si-CLIC3 #4 / Flag-CLIC3

si-con

si-Rab27A+B

MT1-MMP recycled (%) vs time (min)

MT1-MMP recycled (%) vs time (min)

MT1-MMP recycled (%) vs time (min)

MT1-MMP recycled (%) vs time (min)