Role for formin-like 1-dependent acto-myosin assembly in lipid droplet dynamics and lipid storage

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Lipid droplets (LDs) are cellular organelles specialized in triacylglycerol (TG) storage undergoing homotypic clustering and fusion. In non-adipocytic cells with numerous LDs this is balanced by poorly understood droplet dissociation mechanisms. We identify non-muscle myosin IIa (NMIIa/MYH-9) and formin-like 1 (FMNL1) in the LD proteome. NMIIa and actin filaments concentrate around LDs, and form transient foci between dissociating LDs. NMIIa depletion results in decreased LD dissociations, enlarged LDs, decreased hydrolysis and increased storage of TGs. FMNL1 is required for actin assembly on LDs in vitro and for NMIIa recruitment to LDs in cells. We propose a novel acto-myosin structure regulating lipid storage: FMNL1-dependent assembly of myosin II-functionalized actin filaments on LDs facilitates their dissociation, thereby affecting LD surface-to-volume ratio and enzyme accessibility to TGs. In neutrophilic leucocytes from MYH9-related disease patients NMIIa inclusions are accompanied by increased lipid storage in droplets, suggesting that NMIIa dysfunction may contribute to lipid imbalance in man.
In cells, lipid droplets (LDs) are highly dynamic, well-connected with other organelles and actively communicating with one another. LDs undergo major morphological changes depending on the metabolic status of cells. In energy surplus, stimulation of triacylglycerol (TG) storage results in LD enlargement, apparently by both fusion and lipid transfer between adjacent LDs, with the extreme phenotype being unilocular LDs in mature adipocytes. The homotypic fusion of LDs is regulated by Fsp27 enriched at LD contact sites and its activity is controlled by Rab8a. Conversely, TG breakdown by lipolysis is accompanied by LD shrinkage, dissociation and dispersion. This process involves modification of LD resident proteins to allow docking and activation of lipidic enzymes to digest the lipid core, and eventual dispersion of smaller LDs to the cell periphery in a microtubule-dependent fashion. How the initial dissociation of adjacent LDs is achieved, is essentially unknown.

Actin and actin-binding proteins have been reported to be associated with LDs. In murine macrophages, pharmacological disruption of the actin cytoskeleton reduces LD size and lipid storage, and in 3T3-L1 cells, inhibition of actin dynamics through cofilin-1 depletion disrupts adipogenesis and lipid storage. At the organismal level, pharmacological perturbation of acto-myosin turnover in zebrafish affects the recruitment of LDs from the yolk-blastodisc to the animal pole. Interestingly, depletion of NMIIa resulted in increased LDs (about sixfold for LDs between 0.5 and 1.0 μm²) representing the overwhelming majority of LDs and ninefold for LDs in the range of 1.5–2 μm². A similar increase in LD size was observed upon oleic acid administration in NMIIa-depleted cells. Enhanced formation of large LDs was also observed with another NMIIa-targeting siRNA, suggesting that the effect is specific.

To further assess the specificity of the LD enlargement upon NMIIa depletion, we generated U2OS cells stably overexpressing an siRNA-resistant Cherry-NMIIa, or Cherry alone as control. We found that the accumulation of enlarged LDs upon NMIIa depletion was rescued by Cherry-NMIIa overexpression in control conditions as well as upon oleic acid loading. Conversely, NMIIa overexpression in control cells resulted in the reduction of large LDs.

We next explored whether depletion of NMIIa affects neutral lipid storage. We found that the larger size of LDs in NMIIa-depleted cells was accompanied by a significant increase in TG deposition compared to control cells. This effect was obtained upon NMIIa inhibition with blebbistatin and depletion of tropomyosin 4. Interestingly, NMIIa depletion from human cells reduces LD dynamics and alters their clustering propensity, resulting in super-sized LDs and reduced TG hydrolysis. Together, these data suggest that NMIIa assembly with actin in focal points around LDs assists in the dissociation of clustered LDs.

Results

NMIIa depletion results in enlarged LDs and increased TGs. We conducted a proteomic profiling of LD-associated proteins. This revealed several actin-binding proteins (Supplementary Table 1), including NMIIa (encoded by the MYH9 gene), a structural component of contractile actin filaments. NMIIa has been earlier identified in the LD proteome of several organisms, but its functional role at LDs remains unknown.

To investigate the role of NMIIa at LDs, we silenced the protein with human osteosarcoma U2OS cells (Supplementary Fig. 1a–d), a cell line commonly used for studying cytoskeletal dynamics. For systematic analysis of LDs in U2OS cells, we developed automated image analysis tools to quantify LD abundance, size and clustering (Supplementary Fig. 2a). Under normal growth conditions, roughly 100 LDs were detected in these cells using fluorescent LD dyes, with small LDs (below 0.5 μm²) representing the overwhelming majority.

NMIIa localizes transiently to LD dissociation sites. We investigated the localisation of NMIIa at LDs using confocal and three-dimensional structured illumination microscopy (3D-SIM). To facilitate this, we employed mild saponin permeabilization of cells prior to fixation, to reduce the cytoplasmic pool of NMIIa. We found that endogenous NMIIa localized to focal points around LDs visualized by the LD marker peptide HPOS. Cherry stably expressed in U2OS cells (Fig. 2a,b), whereas the periodic labelling pattern of NMIIa along stress fibres was preserved (Fig. 2b). The LD-associated NMIIa patches often localized to two neighbouring LDs or between clusters. On average, LDs were associated with three NMIIa patches and approximately one patch per LD–LD contact site. Additionally, filamentous actin (F-actin) was found at LDs (Fig. 2a,b, Supplementary Fig. 1g–k). Oleic acid administration for 7h roughly doubled the number of LDs (Supplementary Fig. 2b), whereas their relative size distribution was not changed.

To monitor NMIIa dynamics at LDs in living cells, we used Airyscan superresolution microscopy of GFP-NMIIa-expressing cells. This revealed distinct GFP-NMIIa patches transiently concentrating between clustered LDs (Fig. 2d, Supplementary Movie 1; for additional examples, see Supplementary Fig. 4a, Supplementary Movies 2 and 9). In many cases, transient NMIIa accumulation between two LDs was followed by their dissociation (Fig. 2d, Supplementary Movie 1). Plotting of NMIIa and...
LD intensity profiles of dissociating LDs before NMIIa accumulation, upon NMIIa enrichment and after LD segregation, showed a transient increase in NMIIa fluorescence during the partitioning of LDs (Fig. 2e, Supplementary Fig. 4b). Furthermore, we observed transient accumulation of fluorescently tagged actin between dissociating LDs (Supplementary Fig. 4c, d and Supplementary Movie 3), suggesting that NMIIa and actin filaments cooperate in the dissociation of LDs.

**NMIIa affects LD dissociation dynamics and LD clustering.** To address if the loss of NMIIa affects LD dynamics, we monitored the association kinetics of clustered LDs, that is, LDs that were associated with each other. Coherent anti-stokes Raman scattering (CARS) microscopy was employed for label-free imaging of LDs in live cells (Fig. 3a, b, Supplementary Movie 4). This revealed that in control cells, the clustered LDs either stayed associated with each other (static clusters) or were highly...
dynamic, with rapid association and dissociation of the clustered LDs during the 5 min recording (Fig. 3a, Supplementary Movie 4). Interestingly, in NMIIa-depleted cells, static LD associations were more prevalent (Fig. 3b, Supplementary Movie 4). Similar observations were made upon NMIIa inhibition with blebbistatin or disruption of actin polymerisation with cytochalasin D (Supplementary Movies 5 and 6). Analogously to blebbistatin, acute cytochalasin D treatment increased the proportion of larger LDs (Supplementary Fig. 2g). Owing to the similar effects of NMIIa depletion and blebbistatin on LDs, the motor activity of myosin II appears functionally important, and other myosin isoforms, including NMIIb, seem not to fully compensate for the loss of NMIIa.

To obtain further quantitative information on the altered LD association characteristics upon NMIIa depletion, we extended the automated LD detection pipeline with a cluster analysis tool (Fig. 3c). This tool considers LDs to be clustered if the distance between droplet boundaries is smaller or equal to two pixels (0.266 μm), enabling the detection of clusters with a variable number and quantification of average LD sizes as well as LD size heterogeneity within clusters. In control cells loaded with oleic acid, the majority of LD clusters comprised two droplets, followed by a decreasing abundance of clusters containing more droplets (Fig. 3d). Interestingly, NMIIa-depleted cells loaded with oleic acid contained overall less-clustered LDs, with most pronounced reductions for clusters of three and four LDs (Fig. 3d). In parallel, the mean size of clustered LDs increased by ~75% upon NMIIa silencing (Fig. 3e) and their size heterogeneity increased, as evidenced by greater than threefold increase in the s.d. of the size of clustered LDs (Fig. 3f,g). The observed changes in the size of clustered LDs were due to loss of NMIIa, as they were reversed in cells overexpressing the siRNA-resistant Cherry-NMIIa (Fig. 3h,i). These results indicate that NMIIa depletion results in reduced motility of associated LDs and a larger size heterogeneity between associated LDs. Moderate alterations in the balance of LD dissociation/re-association may over time result in prominent LD size changes, with bigger LDs deriving from coalescence of neighbouring LDs, as suggested by the observed fusion between immotile, clustered LDs (Supplementary Fig. 4e,f, Supplementary Movie 7).

**FMNL1 localizes to LD dissociation sites.** Our proteomic analysis also revealed the actin regulatory protein FMNL1 among the LD-associated proteins (Supplementary Table 1). FMNL1 has several activities, such as stimulation of actin polymerisation and bundling of actin filaments, providing a candidate to drive the formation of actin filaments at LDs. We therefore investigated the localisation of endogenous FMNL1 in U2OS cells using Airyscan and 3D-SIM imaging. FMNL1 antibodies revealed punctate cytoplasmic as well as perinuclear immunoreactivity, in agreement with previous studies. To reduce cytoplasmic immunoreactivity, cells were saponin permeabilized pre-fixation, similarly as for NMIIa staining (Supplementary Fig. 5d,e). This highlighted punctate FMNL1 labelling on the surface of LDs (Fig. 4a,b). We found on average three FMNL1 patches per LD and of these approximately half localized to LD–LD contact sites (Supplementary Fig. 5f,g). Interestingly, FMNL1 patches at LDs were closely apposed to NMIIa dots, as visualized by myosin light chain staining (Fig. 4c, Supplementary Fig. 5h). Live cell Airyscan video microscopy demonstrated that GFP-FMNL1 localized transiently to LD dissociation sites (Fig. 4d, Supplementary Movie 8). Of the 155 LD dissociation events quantified, over 60% showed FMNL1 foci between the dissociating LDs (Fig. 4f) and in nearly all of them, FMNL1 was present already before the separation of LDs occurred (Fig. 4g). When a similar analysis was performed from live cell Airyscan videos of GFP-NMIIA expressing cells, we observed about half of the LD dissociation sites to contain NMIIa accumulations (Fig. 4e,f; Supplementary Movie 9). Interestingly, NMIIa seemed to be recruited to the dissociation sites after FMNL1, as in ~1/3 of the events the NMIIa accumulation appeared once LDs had started to separate from each other (Fig. 4g). We also noted that after dissociation some LDs underwent reassociation and that FMNL1 could stay associated with LDs during such events (Supplementary Fig. 6 Supplementary Movies 10 and 11).

To investigate the interrelationship of FMNL1 and actin upon LD dissociation, we performed triple-colour live cell Airyscan imaging of GFP-FMNL1, BFP-Lifeact and LDs in a region below the nucleus, to minimize cytoplasmic background (Fig. 5a). This revealed a transient focal accumulation of GFP-FMNL1 in a LD cluster, followed by dynamic accrual of BFP-Lifeact between dissociating LDs (Fig. 5b,c; Supplementary Movie 12). We also monitored the accumulation of a larger FMNL1 patch apparently driving the separation of neighbouring LDs (Fig. 5d–f; Supplementary Movie 13). This enabled focal intensity measurements of FMNL1 and Lifeact, further substantiating the idea that an increase in FMNL1 intensity preceded that of Lifeact between LDs (Fig. 5f).

**FMNL1 drives actin assembly on LDs and recruits NMIIa to LDs.** Together, these data suggest that FMNL1 can generate actin filaments on LDs, which in turn may recruit NMIIa. In support of this idea, we found a major reduction of NMIIa in LD fractions of FMNL1-silenced cells (Fig. 6a–c; Supplementary Fig. 7f,g). This was accompanied by a decrease of NMIIa immunostaining at LDs upon FMNL1 depletion (Supplementary Fig. 8a–c). This highlighted the potential role for FMNL1 in actin-based regulation of LDs. In agreement with previous studies, this suggests that FMNL1-mediated actin accumulation around LDs may drive LD dissociation via force generation, as recently documented in live cell biology. In turn, this may provide an additional mechanism for LD reassociation and organisation.

**Figure 1 | NMIIa depletion results in enlarged LDs and increased TG deposition.** (a) LD morphology of U2OS cells treated with control or NMIIa siRNA. Cells were stained with LipidTOX green (LDs), DAPI (nuclei) and CellTracker Red (cytoplasm, not shown). (b) Automated LD quantification of U2OS cells treated as in a, ± s.e.m., n = 303 cells for siCtrl and 228 cells for siNMIIa from three independent experiments. (c) Similar as in a with 200 μM oleic acid treatment for 7 h. (d) Automated LD quantification of cells treated as in c, ± s.e.m., n = 293 cells for siCtrl and 204 cells for siNMIIa, from three independent experiments. (e) LD morphology of U2OS cell lines expressing either Cherry or siRNA resistant Cherry-NMIIA (Cherry-NMIIa siRes) and treated with NMIIa siRNA. Cells were stained with LD540 (LDs), DAPI (nuclei) and CellTracker Red (cytoplasm, not shown). (f) Automated LD quantification of cells treated as in e, ± s.e.m., n = 339 for Cherry siCtrl, 261 cells for Cherry siNMIIa, n = 303 cells for Cherry NMIIa siRes siCtrl and 270 cells for Cherry NMIIa siRes siNMIIa, from 3 independent experiments. (g) TG amount of U2OS cells treated with siCtrl or siNMIIa (± s.e.m., n = 6–7 samples from three independent experiments). (h) TG amount of U2OS cells treated with or without 30 μM Blebbistatin for 24 h (± s.e.m., n = 5 samples from two independent experiments). (i) TG hydrolysis in U2OS cells treated with siCtrl or siNMIIa. Cells were treated with 200 μM oleic acid overnight to induce TG storage followed by incubation in complete medium for 8 h to induce TG hydrolysis (± s.e.m., n = 8 samples from four independent experiments). (j) TG synthesis as measured by [3H]-oleic acid incorporation for 2 h in serum free conditions in U2OS cells treated with siCtrl or siNMIIa (± s.e.m., n = 6 samples from two independent experiments). Scale bar 10 μm, 5 μm for insets. Student’s t-test, *P<0.05, **P<0.01, ***P<0.001.
Figure 2 | NMIIa colocalizes with actin on LDs and concentrates between dissociating LDs. (a) Stable U2OS Cherry-HPOS cells were treated with 400 μM oleic acid overnight, saponin permeabilized, fixed and immunostained for NMIIa. Deconvolved confocal images, scale bar 10 μm, 2.5 μm for inset. (b) 3D-SIM of stable U2OS Cherry-HPOS cells treated and stained as in (a) including Alexa 488 Phalloidin to visualize actin. Scale bar 10 μm, 0.5 μm for insets. (c) Electron micrographs of U2OS cells treated with 400 μM oleic acid overnight, immunostained with anti-NMIIa antibodies. Arrows indicate NMIIa localisation, left panel: stress fibres, middle panel: LD surface, right panel: between contacting LDs. Scale bar 200 nm (d) Live cell Airyscan imaging of U2OS cells transfected with GFP-NMIIa for 24 h and loaded with 400 μM oleic acid overnight. Frame rate 633 ms, scale bar 1 μm. Arrows indicate transient GFP-NMIIa accumulation between dissociating LDs (arrowheads). (e) Intensity line profiles to visualize transient GFP-NMIIa accumulation between dissociating LDs at indicated times. This figure is accompanied by Supplementary Fig. 4a,b and Supplementary Movies 1 and 2.
Of note, despite the clear reduction of NMIIa, actin was not reduced in LD fractions of FMNL1-depleted cells (Supplementary Fig. 7h), apparently reflecting the association of also other actin populations, that do not contain NMIIa, to LDs. Finally, FMNL1 depletion resulted in the formation of enlarged LDs analogously to NMIIa depletion (Supplementary Fig. 8a). This was observed despite the fact that FMNL1 depletion also inhibited fatty acid uptake, thereby lowering

**Figure 3 | Altered clustering and association kinetics of LDs in NMIIa-depleted cells.** (a) Live cell CARS microscopy of U2OS cells treated either with siCtrl or siNMIIa, and with oleic acid for overnight. Images were acquired every 2 s for 5 min (scale bar 2 μm for upper panel, 2.5 μm for lower panel). Arrowheads indicate alternating LD associations (upper panel) and stable LD associations (lower panel). (b) Quantification of LDs that stay associated during 5 min, for videos acquired as in a, oleic acid load was for 8 h to overnight. ± s.e.m., n = 3 independent experiments, at least 290 LD associations were quantified per treatment. (c) Example of automated LD cluster detection (scale bar 2.5 μm). Arrows indicate detected LD clusters. (d) Automated LD cluster analysis of U2OS cells treated with siCtrl or siNMIIa, and oleic acid for 7 h. Cluster analysis was performed on the same image sets as in Fig. 1d. Number of clusters detected per 100 LDs per cell were quantified for cluster sizes containing 2, 3, 4 and 5 LDs. ± s.e.m. n = 3 independent experiments. (e) Automated quantification of mean LD size in clusters. (f) Automated quantification of LD size heterogeneity in clusters (for e and f, ± s.e.m. n = 258 cells for siCtrl and 134 cells for siNMIIa, from three independent experiments). (g) Schematic representation for the quantification of LD size heterogeneity within clusters. (h) Quantification of mean LD size in clusters in U2OS cells stably overexpressing (o.e.) Cherry or Cherry-NMIIa-siRes treated with siCtrl or siNMIIa, and oleic acid for 8 h. Quantification was performed from the same data set as in Supplementary Fig. 2e (± s.e.m., n = 224 cells for siCtrl U2OS Cherry, 160 for siNMIIa U2OS Cherry, 206 for siCtrl U2OS Cherry-NMIIa siRes and 151 for siNMIIa U2OS Cherry-NMIIa siRes). (i) Quantification of LD size heterogeneity from the same data sets as in h. Student’s t-test *P < 0.05, **P < 0.001.
Figure 4 | FMNL1 localizes to LDs and coincides with LD dissociation sites. (a) Airyscan microscopy of stable U2OS Cherry-HPOS cells treated with 400 μM oleic acid overnight, saponin permeabilized, fixed and immunostained for FMNL1. Scale bar 10 μm, 2.5 μm for inset. (b) 3D-SIM of stable U2OS Cherry-HPOS cells treated and stained as in (a). Scale bar 1 μm. (c) 3D-SIM of stable U2OS Cherry-HPOS cells treated with 400 μM oleic acid overnight, saponin permeabilized, fixed and immunostained for FMNL1 and myosin light chain. Scale bar 10 μm, 1 μm for inset. Arrows indicate close proximity of FMNL1 and myosin light chain on LD surface. (d) Live cell Airyscan microscopy of GFP-FMNL1 and LDs in U2OS cells treated with 200 μM oleic acid overnight, images were acquired every 925 ms. Scale bar 0.5 μm. Arrow indicates GFP-FMNL1 accumulation between dissociating LDs (arrowheads). (e) Live cell Airyscan microscopy of GFP-NMIIa and LDs in U2OS cells treated with 400 μM oleic acid overnight, images were acquired every second. Scale bar 0.5 μm. Arrow indicates GFP-NMIIa accumulation between dissociating LDs (arrowheads). (f) Quantification of LD dissociation events positive for FMNL1 or NMIIa from videos acquired as in (d,e). 155 LD dissociation events were assessed from 7 GFP-FMNL1/LD videos and 136 LD dissociation events from 13 GFP-NMIIa/LD videos. (g) Timing of FMNL1 and NMIIa recruitment to LD dissociation sites. LD dissociation sites positive for FMNL1 or NMIIa were assessed for whether FMNL1 or NMIIa associated with the site before or only during the dissociation event. The same datasets as in (f) was used for quantification. Student’s t-test **P < 0.01. See also Supplementary Movies 8 and 9.
TG storage (Supplementary Fig. 8b,c). Together, these data suggest that FMNL1 mediates LD localisation of NMIIa, possibly by generating specific actin filaments to which NMIIa binds.

To provide more direct evidence for actin filament assembly on LDs and the potential role of FMNL1 in this process, we designed an in vitro assay, which allows the monitoring of actin polymerisation on isolated LDs using total internal reflection fluorescence (TIRF) microscopy. LDs were isolated from oleic acid-loaded U2OS cells, labelled with LipidTOX green, and then mixed with rhodamine actin and unlabelled actin in a TIRF reaction chamber (Fig. 6d). Actin filament assembly was monitored by video TIRF microscopy (Fig. 6d,e). We found that under these conditions ~6% of isolated LDs associated with actin filaments and displayed time-dependent growth of actin filaments from them (Fig. 6e–g). That only a small fraction of LDs displayed actin nucleation may result from partial dissociation of FMNL1 from LDs during isolation or absence of soluble FMNL1-interacting proteins in the in vitro system. Thus, the process may be more efficient in living cells. Addition of the formin inhibitor SMIFH2 (ref. 22) or use of LDs isolated from FMNL1-depleted cells in the in vitro assay blunted actin filament association with LDs (Fig. 6g).

MYH9RD patient neutrophils display altered lipid storage. Mutations in the human MYH9 gene cause a rare autosomal dominant disorder generally referred to as MYH9-related disease (MYH9-RD), with tens of individual disease causing mutations identified23. MYH9-RD is characterized by NMIIa inclusion bodies in neutrophilic leucocytes, thrombocytopenia with giant platelets and multiorgan symptoms, including

![Figure 5](https://example.com/figure5.png)
sensorineural hearing loss, renal failure and presenile cataract, the pathogenesis of which are incompletely understood. To investigate whether cells from patients with MYH9-RD display a LD-related phenotype, we isolated peripheral blood neutrophilic leucocytes from two patients with MYH9-RD carrying a point mutation in the C-terminal non-helical tail of NMIIa (S. Ryhänen, personal communication) and two healthy individuals. These patients present with marked macrothrombocytopenia and several family members have been diagnosed with hearing loss, in accordance with the site of the mutation. By NMIIa immunostaining, the diagnostic NMIIa inclusion bodies of the disease were evident and the overall NMIIa immunoreactivity was decreased in the patient neutrophils (Fig. 7a,b). Under basal conditions (fasting blood samples, no lipid loading in vitro), both control and patient neutrophils displayed few LDs (approximately seven LDs per cell) (Fig. 7c,d). Remarkably, upon 1 h of oleic acid loading in vitro, LD accumulation was more pronounced in the patients’ neutrophils, resulting in roughly twofold more LDs compared with controls (Fig. 7d,e). In parallel, there was a tendency for the enlargement of LDs in the patients’ cells, especially in the bigger LD size classes (Fig. 7e). Thus, the LD phenotypes in the patient neutrophils appeared partly reminiscent of those observed in NMIIa-silenced U2OS cells. Together, these findings provide the first evidence that NMIIa dysfunction in man causes a lipid imbalance, possibly contributing to the pathogenesis of MYH9-RD. For instance, neutrophil LDs can modulate the immune response, as impaired LD lipolysis was found to be accompanied by reduced lipid mediator release.

Discussion

Actin-binding proteins are prominent constituents of the LD proteome but their specific roles on LDs are not well understood. In this study, we describe a functional interplay of the actin-polymerising factor FMNL1 and NMIIa, a force generating protein, in LD dynamics. NMIIa is generally known as a major constituent of contractile stress fibres, regulating cell

Figure 6 | FMNL1 regulates NMIIa recruitment to LDs and actin assembly on isolated LDs. (a) FMNL1 depletion reduces NMIIa in LD fractions. Stable U2OS Cherry-HPOS cells were treated with siControl or siFMNL1 and with 400 μM oleic acid overnight. Western blots for NMIIa and Perilipin-2 (Plin2) are shown. (b,c) Quantification of NMIIa abundance in LD fractions relative to Plin2 (b) or neutral lipid content (c), ± s.d. from two independent experiments. (d) Schematic representation of TIRF microscopy based monitoring of actin assembly on isolated LDs. LDs were isolated by density gradient centrifugation from U2OS cells treated with siCtrl or siFMNL1 and loaded with 400 μM oleic acid overnight. A mix of 0.15 μM unlabelled non-muscle actin, 0.065 μM Rhodamine non-muscle actin and 0.43 μM profilin was polymerized in the presence of LDs. Actin polymerisation was imaged by time-lapse TIRF microscopy for 15 min at 100 ms intervals. (e) Representative example of actin filament assembly on an isolated LD. Scale bar 2.5 μm. (f) Representative images from actin assembly studies with LDs isolated from control cells (left image), siFMNL1-treated cells (centre image) or LDs from control cells incubated with formin inhibitor (SMIFH2) (right image). Scale bar 5 μm. Arrows indicate LDs with associated actin filaments. (g) Quantification of nascent actin filaments associated with LDs. LDs stably associated with actin filaments were quantified and normalized to the total LD number per image field. ± s.e.m., n = 11 assays for siCtrl, 11 assays for siFMNL1 and 7 assays for formin inhibition. Student’s t-test *P<0.05, **P<0.01, ***P<0.001.
Migration and focal adhesion stability. NMIIa binds actin filaments and ATP hydrolysis evokes a conformational change, resulting in pulling along actin filaments and force generation. Recent evidence indicates that NMIIa can also act on specialized actin filament structures, for instance, during mitochondrial fission.

We found that NMIIa transiently accumulates at sites where LDs separate from one another. LDs are known to undergo homotypic associations and dissociations, resulting in the formation of dynamic LD clusters. Our data show that NMIIa has a major impact on cellular LDs, with NMIIa depletion resulting in enlarged LDs and increased TG storage owing to impaired lipid breakdown. We postulate that impaired dissociation/prolonged association of LDs enhances their coalescence, either via direct fusion (Supplementary Fig. 4e,f; Supplementary Movie 7) and/or via lipid transfer. This would result in an increased size heterogeneity of LDs and a net consumption of LDs within a cluster, with a concomitant reduction of clusters. Indeed, NMIIa silencing reduced LD dissociations. In parallel, the LD clustering propensities were altered: there were fewer LD clusters and in these big LDs often associated with small ones. This is strikingly different from control cells where similarly sized LDs formed clusters.

NMIIa requires a preformed actin filament to exert its function. But how could an actin filament be generated at LD dissociation sites? We demonstrate that FMNL1, a protein with actin polymerisation activity, localizes to LD dissociation sites. It can thus provide a seed for acto-myosin assembly, as depletion of FMNL1 abolishes NMIIa association with LDs. Furthermore, we found that isolated LDs have the ability to induce actin filament assembly in vitro. This activity was dependent on FMNL1, as pharmacological inhibition of formins or depletion of FMNL1-blunted actin filament assembly on isolated LDs. Based on these observations, we suggest that FMNL1 localizes to LD dissociation sites to nucleate actin filaments that are functionalized by NMIIa, providing a force to assist in LD dissociation. This idea is supported by the temporal association of the actin-binding proteins with LDs, with FMNL1 accumulation typically occurring between closely apposed LDs before dissociation, potentially defining LD dissociation sites. Although most dissociating LDs also harbour NMIIa, it may also be recruited to the site once LDs are moving apart.

**Figure 7 | Effect of MYH9-RD on LDs in primary human neutrophils.** (a) Visualisation of NMIIa inclusions (indicated by arrowheads) in MYH9-RD patients (Pt1, Pt2) neutrophils. Scale bar 5 μm. Image intensities were adjusted to ensure visibility of NMIIa inclusions in patient cells. (b) Quantification of NMIIa intensity in control and patient neutrophils. ± s.e.m. n = 6–22 cells for each. (c) LD staining (LD540) of control and patient neutrophils in basal conditions and upon loading with 10 μM oleic acid for 1h. Scale bar 10 μm. (d) Quantification of LD numbers in (c). ± s.e.m. n = 40 cells for Ctrl 1; 38 cells for Ctrl 2; 54 cells for Pt 1; 35 cells for Pt 2. (e) Size distribution of LDs upon oleic acid loading, same data set as in (d). The inset displays the relative amount of LDs larger than 0.032 μm² ± s.d. Student’s t-test, *P < 0.05, **P < 0.001.
Considering that LDs also undergo rapid reassociations, an alternative, although not mutually exclusive explanation is that FMNL1, in concert with actin and NMIIa, prevents the reassociation and fusion of LDs. Both mechanisms, promotion of LD dissociation and prevention of LD reassociation, highlight the importance of LD dynamics in controlling the coalescence and surface to core ratio of LDs, and thereby the accessibility of modifying enzymes to core TGs.

**Methods**

**Antibodies, dyes and reagents.** Rabbit anti-NMIIa (Covance, PRB-440P Immunofluorescence (IF) 1:500, Immunoblot (IB) 1:3,000), mouse anti-alpha tubulin (Sigma Aldrich, T6074, IB 1:20,000), rabbit anti-NMIIa (Sigma Aldrich, HPA008549, IB 1:1,000), rabbit anti-FMNL1 (BD, Billadeau, Mayo Clinic, Rochester, Minnesota, IF 1:50)24, mouse anti-myosin light chains (Sigma Aldrich, M4401, IF 1:2000), Alexa488 goat anti-rabbit, A10108; Alexa568 goat anti-rabbit, A10111; Alexa647 donkey anti-rabbit, A31573 (all Thermo Fisher, 1:200); HCS LipidTOX green neutral lipid stain, H34475, (live cell 1:1,000, fixed cells 1:10,000); Alexa488 Phalloidin, A12379, (132 nM) all Thermo Fisher, LDS40 (Princeton BioMolecular Research, 0.1 μg ml⁻¹); Cell culture reagents and general reagents were purchased from GibCo/Thermo Fisher, Lonza and Sigma-Aldrich.

**siRNAs, plasminid and mutagenesis.** siRNAs targeting NMIIa and FMNL1 were purchased from Qiagen and control siRNA from Sigma-Aldrich. Target sequence siControl: 5'-CGTAATTCGGATACATTCGCA-TCA-3' (ref. 30); siNMIIa: 5'-TGCAAGCCCACGCAGAGGAC-3'; siFMNL1: 5'-TCGCTGTCGCCCCTGCATCTTATT-3'; siNMIIa-3Prime: 5'-CACGGAGATGGAGGACCTTAT-3'. Plasmids were transfected using Lipofectamine LTX (Thermo Fisher) with 50 nM siRNA. Oleic acid loading was performed during the siRNA treatment period. For generation of siRNA-resistant NMIIA we first generated two PCR fragments including the siRNA resistant sequence in the corresponding -3Prime and -3H1 restriction sites to generate GFP-NMIIa-siRes.

For generation of siRNA-resistant FMNL1 we first amplified plasmid pCAGGS-FMNL1 with oligonucleotides: FMNL1FWNhe1: TGAAGCGATTGGCAAGATATTCAGCAATATCC-GCATCAGCACGCAGA-3' and FMNL1RV Nhe1: 5'-GAGAGCTAGCATGGGCAACGCGGCCGGCA-3' and cloned into pmCherry-C100, pmTagBFP2-N (from V. Verkhusha) via BglEI and NotI restriction sites to generate GFP-NMIIa-siRes. mCherry-NMIIa-siRes. For the generation of Cherry-HPOS, oligonucleotides were synthesized, annealed and inserted into pmCherry-C100.

**LD isolation and mass spectrometry.** Raw 264.7 cells (received from Vesa Olkkonen, Minerva Foundation Institute for Medical Research, Helsinki, Finland) were cultured in DMEM with 10% FBS, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), L-glutamine (2 mM) and penicillin/streptomycin (100 U ml⁻¹). Cells were treated with 400 μM oleic acid or 35 μg ml⁻¹ acetylated low density lipoprotein in DMEM with 5% lipoprotein deficient serum for 24 h. LD isolation was performed similar to previously described22. The treatments were combined for LD isolation to maximize the yield. Cells were washed with cold phosphate buffered saline (PBS) and resuspended in 3.5 ml hypotonic lysis buffer (HL: 20 mM Tris pH 7.4, 1M ethylenediaminetetraacetic acid), and incubated on ice for 10 min. Cells were disrupted by repetitive passing through a 25 g needle, 3 ml of cell suspension was transferred to a Beckmann polyallomer tube (14 x 95 mm) (No. 333174) and mixed with 1.5 ml 60% sucrose solution, overlayed with 4 ml 5% sucrose solution and 4 ml HLM Buffer. Samples were centrifuged at 28,000 g with a SW40 rotor for 2 h. The LD fraction was recovered by tube slicing and subjected to liquid chromatography–mass spectrometry (LC-MS/MS). For LC-MS/MS, samples were prepared as follows: the cytosolic proteins were reduced with 2 mM TCEP (Sigma–Aldrich), alkylated with 10 mM iodoacetamide (Sigma–Aldrich), and the proteins were digested to peptides by trypsin (Promega, Madison, WI). After overnight incubation at 37°C, samples were quenched with 10% TFA, purified with C18 Micro SpinColumns (Harvard Apparatus, Holliston, MA) and re-dissolved in 30 μl 0.1% TFA, 1% CH3CN. The LC-MS/MS samples were loaded on a 0.17 mm i.d. EASY-nLC nanoflow system (Thermo Scientific) connected to a Velos Pro-Orbitrap Elite hybrid mass spectrometer (Thermo Scientific) using the Xcalibur version 2.7.1. The peptides were separated using 10 cm analytical column (Thermo Fisher Scientific) with 60 min gradient ranging from 5 to 35% buffer B (0.1% TFA in acetonitrile in 50% acetonitrile in 0.1% TFA in acetonitrile). The precursor and fragment ions were then extracted and subjected to data-dependent mass spectrometric analysis. Error tolerances on the precursor and fragment ions were set to 15 ppm and 0.6 Da, respectively. Database searches were limited to fully tryptic peptides with maximum one missed cleavage. For peptide identification the FDR was set to <5%.

**LD isolation and actin assembly on isolated LDs.** U2OS cells (6–7 cm dishes) were treated overnight with 400 μM oleic acid in culture medium, EDTA in HLM buffer was replaced with EGTa. LD isolation was performed as described above and LD fractions were retrieved from the top with a 1 ml pipette. In vitro LD imaging was performed on a Nikon Eclipse Ti-E N-STORM microscope, equipped with Andor iXon EMCCD camera and 100 APO TIRF oil objective NA 1.49, a 561 nm laser line was used for visualisation of Rhodamine actin and 2% DMSO, the actin polymerisation was followed in 0.1 s intervals for 15 min after addition of the reaction into the imaging chamber.

**LD preparation for western blot analysis.** U2OS Cherry-HPOCs cells were seeded in 2–4 10 cm dishes and transfected with siControl or siFMNL1. After 24 h cells were incubated with 400 μM oleic acid in control medium for an additional 24 h. Cells were washed 2 x with Dulbecco’s phosphate-buffered saline (DPBS) and then scraped into 5 ml DPBS. Cells were pelleted by centrifugation at 730 g for 5 min and resuspended in 900 μl HLM buffer. Cells were broken by repetitive passing through a 25 g needle, and 750 μl of the suspension was mixed with 750 μl HLM buffer with 60% sucrose, overlayed with 1 ml HLM with 5% sucrose followed by 1.5 ml HLM buffer. LDs were floated by centrifugation at 28,000 g for 2 h. The LD fraction, 10% was desalted by EGTA, and the rest was subjected to acetone precipitation with 10 x volume of ~75°C acetone. Samples were incubated at ~20°C degree overnight and centrifuged at 3,300 g for 100 min. Proteins were resuspended in 2 x loading buffer by sonication and subjected to western blot analysis. Original scans of the respective western blots is shown in Fig. 6a can be found in Supplementary Fig. 9.

**Automated LD analysis.** U2OS cells were cultured on coverslips or 96 well Screenstar plates (Greiner) and incubated with or without oleic acid for 7 h. Cells were treated with 2 μM CellTracker Red for 30 min to 1 h in culture medium with or without oleic acid, washed 3 x with PBS and fixed with 4% paraformaldehyde in 250 mM HEPES, 1000 mM CaCl, 100 mM MgCl₂ pH 7.4. Cells were washed 3 x with PBS and stained with 1 μg ml⁻¹ 4,6-diamidino-2-phenylindole (DAPI) and 100 nM 1,1'-di-4-phenyl-5(6)-carboxyfluorescein (FM4-64) in PBS for 30 min at room temperature (RT), washed 3 x with PBS and mounted with PBS and PBA and epoxy glue. For every treatment 10 image stacks were acquired for DAPI, LD and cell tracker.
channels using a Nikon Eclipse Ti-E inverted microscope equipped with a 60 × PlanApo VC oil objective. The objective was equipped with a 1.40 numerical aperture. Confocal and superresolution light microscopy was performed using a confocal microscope (Leica TCS CRSP) or a confocal microscopy (Leica TCS CRSP) with a 63 × 1.40 Plan-apochromat objective. For Airyscan superresolution microscopy, cells were imaged with a Leica TCS CRSP microscope equipped with an Airyscan detector using a 63 × Plan-apochromat objective, NA 1.4. Confocal microscopy was performed using a confocal microscope (Leica TCS CRSP), a confocal microscope (Leica TCS CRSP) with a 63 × 1.40 Plan-apochromat objective, NA 1.4. Airyscan microscopy was performed using a confocal microscope (Leica TCS CRSP) equipped with an Airyscan detector using a 63 × Plan-apochromat objective, NA 1.4. Airyscan microscopy was performed using a confocal microscope (Leica TCS CRSP) equipped with an Airyscan detector using a 63 × Plan-apochromat objective, NA 1.4. The Airyscan detector was acquired before each image or video acquisition and images were Airyscan processed using the Zeiss Zen software package. 3D-SIM was performed on an Airyscan microscope equipped with a Leica TCS CRSP confocal microscopy. Localisation of NMIIa at LDs was assessed in 11 independent experiments; localisation of NMIIa at LDs upon FMNL1 depletion in three independent experiments; colocalisation of NMIIa and phallolidin at LDs in eight independent experiments; FMNL1 localisation at LDs in eight independent experiments; localisation of FMNL1 and myosin light chain at LDs in three independent experiments; localisation of myosin light chain at LDs in five independent experiments.

Lipid extraction, analysis and TG hydrolysis. Lipid extraction was performed as described previously66. Cells were washed 3 × with PBS and scraped into 2% sodium chloride and lipids were extracted with chloroform:methanol in a 2:1 ratio. Solvents were evaporated and lipids dissolved in 2:1 chloroform: methanol for application to thin layer chromatography (TLC). TLC was performed with hexane:dichloroethylenic acid 80:20. TLC plates were stained with copper sulfate and developed by charring. TG content was quantified and normalized to protein content measured by Bio-Rad protein determination. For the TG hydrolysis assay, sCtnl and sINMFA-treated U2OS cells were loaded with 200 μM oleic acid for 24 h, with or without inhibition of cholesterol ester synthesis with Synthesis inhibitor. Cells were washed with PBS and incubated with a 2:1 chloroform:methanol solution for 8 h. Lipids were extracted before or after the 8 h chase in normal growth medium. TG content was quantified and used to calculate the percent decrease in TG before the 8 h chase. In cells treated with control siRNA Tgrs were reduced on average by 45% during the 8 h chase.

Western blot analysis. Cells were washed 3 × with PBS and lysed with Tris-buffered saline (TBS)/1% Triton X-100. Lysates were incubated on ice for 10 min, vortexed and centrifuged for 10 min at 16,000 g at 4 °C. Cleared supernatants were mixed with loading buffer (50 mM Tris pH 6.8, 2% sodium dodecyl sulfate (SDS), 1% β-mercaptoethanol, 5% glycerol, final concentrations) and equal protein amounts were separated on SDS-polyacrylamide gels and transferred to Immobilon polyvinylidene fluoride (Millipore) membranes. For Odyssey antibody detection, blots were blocked with Odyssey Blocking buffer/PBS with 0.1% Tween and incubated for 45 min at room temperature. Blots were washed with Odyssey Blocking buffer/PBS with 0.1% Tween and incubated for 2 h at RT. Blots were washed with TBS/0.1% Tween and incubated with secondary antibodies in Odyssey Blocking Buffer/PBS with 0.1% Tween and 0.01% SDS for 30 min at RT. Blots were washed with TBS/0.1% Tween and PBS and scanned with an Odyssey CLx detection system. For enhanced chemiluminescence (ECL) detection, blot was blocked with TBS/0.1% Tween with 5% skim milk for 2 h at RT. Primary antibodies were incubated overnight at 4 °C. Blots were washed with TBS/0.1% Tween and incubated with secondary antibodies in blocking buffer for 30 min. Blots were washed with TBS/0.1% Tween, PBS and distilled water. ECL was performed with Clarity Western ECL substrate (Bio-Rad).
Pre-embedding immuno-EM. U2OS cells were loaded with 400 µM oleic acid overnight and fixed with 4% paraformaldehyde in MES-sucrose buffer (10 mM MES/138 mM KCl/3 mM MgCl2/2 mM EGTA/11.14% sucrose) for 2h. The cells were then permeabilized with 0.01% saponin in 0.1% BSA/0.1 M Na-P04 pH 7.4 for 8 min, labelled with the rabbit anti-NMIIa antibody (Covance, PRB-440 P 1:50) for 2h followed by the nano-gold-conjugated anti-rabbit Fab-fragments for 1 h (Nanoprobes, 2004, 1:60), post-fixed in 10% glutaraldehyde, and quenched in 50 mM glycine. The nano-gold particles were intensified using the HQ SILVER Enhancement kit according to the manufacturer’s instructions (Nanoprobe) followed by gold toning in subsequent incubations in 2% NaAcetate, HAUCl4 and 0.3% Na2S2O3+H2O. The cells were treated with 1% Oso4, supplemented with 15 mgml−1 K[Fe(CN)]6, and flat-embedded in Epon as described earlier. Ultrathin sections were cut using Leica UCT microtome, picked on piofolo coated single slot grids, post-stained with uranyl acetate and lead citrate, and observed with the Jeol JEM-1400 microscope at 80 kV. Images were acquired with the Gatan Orics SC1000B camera.

Neutrophil isolation and lipid loading. Freshly isolated peripheral blood from MYH9-RD patients and from healthy individuals was used for neutrophil isolation using Polymorphrep (Axis-Shield) and contaminating erythrocytes were lysed using 155 mM NH4Cl, 12 mM NaHCO3, 0.1 mM EDTA. Neutrophils were resuspended in Hank’s balanced salt solution without calcium and magnesium and resuspended in Hank’s balanced salt solution without calcium and magnesium and incubated with 10 mM MES/138 mM KCl/3 mM MgCl2/2 mM EGTA/11.14% sucrose for 2h. Neutrophils were then loaded with 400 µM oleic acid and incubated with 10 mM MES/138 mM KCl/3 mM MgCl2/2 mM EGTA/11.14% sucrose for 2h.

Data availability. The proteome of cholesteryl-ester-enriched versus triacylglycerol-enriched lipid droplets.

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**Author contributions**
S.P. designed and performed experiments, analysed data and wrote the manuscript. G.G. performed in vitro actin experiments and analysed data. P.H. designed automated lipid droplet analysis tools. S.J.R. provided patient samples and J.P. performed neutrophil isolations. V.T.S. performed 3D-SIM imaging and L.K. electron microscopy. M.V. performed lipid droplet proteomics. P.L. designed experiments and analysed data, E.I. designed experiments, analysed data and wrote the manuscript. All authors commented on and approved the manuscript.

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