investigation (Jucker and Walker, 2011). The current model stipulates that aggregation in disease is initiated by a protein seed that forms a template for further protein aggregation (Jucker and Walker, 2013). Support for this model comes from research showing that the exogenous addition of minute

a given pathway is due to chance alone. Our threshold of the *p*-value for association was set to 0.01.

Western Blot

Nine μl Urea/SDS samples (insoluble proteins) were loaded on a 4–12% gradient gel. The membrane was probed with anti-14-3-3 (1:5000, SC-1657, Santa Cruz Biotechnology). The quantification was done with ImageJ.

RESULTS

Protein Aggregates Formed during Normal *C. elegans* Aging Seed Aβ Aggregation *In Vitro*

TABLE 1 | Ingenuity canonical pathways identified in the set of late- or early-aggregating proteins and their association with minor components found in AD pathological aggregates [–log(*p*-value) > 2].

	log(*p*-value)	
Ingenuity canonical pathways	**Late-aggregating proteins**	**Minor components**
Protein ubiquitination pathway	6.82	2.57
Aryl hydrocarbon receptor signaling	4.93	3.19
Glutathione-mediated detoxification	4.75	
EIF2 signaling	4.54	
tRNA charging	4.23	
mTOR signaling	3.96	
LPS/IL-1 mediated inhibition of RXR function	3.67	
Regulation of eIF4 and p70S6K signaling	3.66	
PI3K/AKT signaling	3.23	5.37
ERK/MAPK signaling	3.11	3.18
Citrulline biosynthesis	3.09	
Calcium transport I	2.89	7.47
Death receptor signaling	2.79	
Superpathway of citrulline metabolism	2.59	
NRF2-mediated oxidative stress response	2.38	4.96
Xenobiotic metabolism signaling	2.31	2.92
14-3-3-mediated signaling	2.25	13.4
Endoplasmic reticulum stress pathway	2.24	
Glutathione redox reactions I	2.20	
Lipid antigen presentation by CD1	2.06	
	Early-aggregating proteins	**Minor components**
EIF2 signaling	5.68	
Protein ubiquitination pathway	5.22	2.57
Cell cycle control of chromosomal replication	4.87	
Aryl hydrocarbon receptor signaling	4.78	3.19
NRF2-mediated oxidative stress response	4.01	4.96
Creatine-phosphate biosynthesis	3.84	
Glutathione-mediated detoxification	3.73	
Leucine degradation I	3.29	
Xenobiotic metabolism signaling	3.08	2.92
Unfolded protein response	2.93	
eNOS signaling	2.52	
Aldosterone signaling in epithelial cells	2.42	2.05
Glutamine biosynthesis I	2.42	
Prostate cancer signaling	2.25	
Choline degradation I	2.12	

Including early-aggregating proteins that continued to aggregate strongly at day 10 and day 14.

patients' brains. We analyzed insoluble extracts from di□erent stages of adulthood to determine when the seeds triggering Aβ aggregation appear. For this, we chose four time points: young animals (day 2), early middle-aged animals (day 6, at the end of reproduction in wild-type animals), late middle-aged animals (day 10, 30% of the population has died) and old animals (day 14, 50% of the population has died). To collect large numbers of individuals for all time points, we cultured *C. elegans* in liquid. The whole procedure was performed four times to obtain four di□erent biological replicates. In order to compare the seeding activities of all insoluble extracts measured in two separate assay plates, we included two standard extracts: a soluble extract as negative control and an insoluble extract from transgenic *C. elegans* overexpressing Aβ as positive control. The relative seeding activity was calculated by normalizing the absolute lag times measured for each biological replicate to those of the standards with 0% being the lag time of the negative control and 100% being the lag time of the positive control. Sypro Ruby staining of insoluble proteins on an SDS gel showed accelerated protein aggregation with increasing age, especially between day 10 and day 14 (**Figure 1B**). Evaluation of the relative seeding activity did not reveal a significant increase in seeding between day 2 and day 6 (**Figure 1C**, *p* D 0.57). Instead, we found a large increase in seeding activity at day 10 (*p* D 0.0086

TABLE 2 | Minor components of AD pathological aggregates identified as late- or early-aggregating proteins in *C. elegans*.

C. elegans entry name	*C. elegans* Uniprot ID	*C. elegans* gene name	*Homo sapiens* protein name (homologs)
Late-aggregating proteins			
14331_CAEEL[x;†]	P41932	par-5	14-3-3 proteins ß/α; δ
C1P636_CAEEL[x],	C1P636	uba-1	Ubiquitin-like modifier-activating enzyme 1
G4S034_CAEEL[x],	G4S034	spc-1	Spectrin α-chain, non-erythrocytic protein 1
LMN1_CAEEL[x],	Q21443	lmn-1	Lamin A/C
Q27473_CAEEL[‡],	Q27473	nex-3	Annexin A5
H4_CAEEL[‡]	P62784	his-1	H4 histone family, member C
AT1B3_CAEEL[‡]	Q9XUY5	nkb-3	Sodium/potassium-transporting ATPase beta-1 chain
G5EEG8_CAEEL[x]	G5EEG8	frm-1	Band 4.1-like protein 1
Q95XP6_CAEEL[x]	Q95XP6	mca-3	Plasma membrane Ca^{CC}-transporter ATPase 1
G3P1_CAEEL[‡]	P04970	gpd-1	Glyceraldehyde-3-phosphate dehydrogenase
Early-aggregating proteins			
GSTP1_CAEEL[x;C]	P10299	gst-1	Glutathione *S*-transferases Mu 3, Mu 5
DYHC_CAEEL[x;†]	Q19020	dhc-1	Cytoplasmic dynein 1 heavy chain 1
HSP90_CAEEL[x;†]	Q18688	daf-21	Protein HSP90-β
G5ECP9_CAEEL[x]	G5ECP9	vab-10	Plectin
HSP7A_CAEEL[‡]	P09446	hsp-1	Heat shock cognate 71 kDa protein
KARG2_CAEEL[‡]	Q27535	ZC434.8	Creatine kinase B-type

[x] *Minor components identified in both amyloid plaques and NFTs in Ayyadevara et al. (2016a).*
[†] *Minor components identified in amyloid plaques in Liao et al. (2004).*
[‡] *Minor components identified in NFTs in Wang et al. (2005).*
Minor components identified as late-aggregating proteins in both day 10 and day 14.
[C] *Minor component identified as early-aggregating protein with a high increase in aggregation at both day 10 and day 14.*

compared to day 2). The seeding potential was not further increased in insoluble extracts from 14-day-old animals. When extrapolated to human aging, the appearance of heterologous protein aggregate seeds for Aβ aggregation at the later stages of life rather than early middle age would be consistent with the late-onset of AD dementia.

Identification of Early- and Late-Aggregating Proteins during Aging

The results from the *in vitro* assay imply that changes in aggregation between day 6 and day 10 are responsible for seeding. We performed quantitative mass spectrometry using the stable-isotope iTRAQ reagents to identify these changes with two independent biological repeats. We identified 845 quantifiable aggregation-prone proteins (Supplementary Table 3). Of these, 460 were identified in our previous study (David et al., 2010). This large overlap attests to the quality of the present mass spectrometry analysis. After normalization, we ranked the proteins depending on their relative change in aggregation levels and we focused on proteins with the highest change in a(g)29atpresene
in4843d(t)-4(he)-6Cday co2mpared48434oayday
lat3 dy40278(proteins409 [(a)1((40278(pre)2(sent)0278(in409 4(t)-4(he)027)-6834027h75)-4(he)4093(hig)-6(he)28itd)]TJ 0 -11.457 Td 7(of602.
in4159(repe)-5(a41591(plogic)-3atte)2.(a4159Signntiic)-3ntmply4159amo41039(t)-4(he)2(se41598(e)-5(art3)-531(dy)ng)ary
protei,ld theate ofaggregationesedincreaedctngmple

FIGURE 3 | Late-aggregation-prone protein PAR-5 accelerates Aβ toxicity in *C. elegans*. (A) Left: representative immunoblot detecting PAR-5 (14-3-3) in insoluble protein extracts from different ages. Right: immunoblot quantification of PAR-5. Band intensities were normalized to day 14. *N* = 4 (biological repeats), SEM depicted. **(B)** Paralysis levels of worms that overexpress PAR-5 (PAR-5 OE), Aβ (Aβ OE) or both (double transgenic, Aβ OE + PAR-5 OE). Shown are the percentages of worms paralyzed at different days. The numbers in the bars represent the total numbers of worms analyzed. Fisher's exact test: Aβ OE vs. Aβ OE + PAR-5 OE, day 3 $p = 0.032$ and day 4 $p = 0.019$.

tested without any enrichment for insoluble proteins. Here, we measured detergent-insoluble protein extracts from young (2–3 months) and aged (18–20 months) mouse brains. Compared to young extracts, insoluble protein extracts from aged mouse brains led to a significant decrease of the lag time preceding Aβ aggregation (**Figure 4**, *p* D 0.0073, Supplementary Figure 4, *p* D 0.019). No further increase in seeding potential was detected when examining insoluble extracts from 25 to 28 month old mice (**Figure 4**, *p* D 0.0067 compared to 2–3 months). This is consistent with the similar seeding activity of extracts from late-middle aged and old *C. elegans*. For comparison, we included the insoluble extract from an APP23 transgenic mouse which contains Aβ seeds and the soluble extract from an aged wild-type mouse brain.

Collectively, these results demonstrate that cross-seeding of Aβ

that higher levels of age-dependent seeds accumulate in these regions and induce tissue-specific vulnerability to disease protein aggregation in AD. Overall, it will be important to determine which proteins and in which locations have seeding activity.

Our proteomic timeline analysis highlights several minor plaque or NFT components that should be prime candidates as initiators of Aβ or tau seeding events to be investigated in future studies. One of these candidates, 14-3-3, has already been confirmed as a heterologous seed for tau aggregation (Li and Paudel, 2016). Importantly, we observed that 14-3-3 overexpression accelerated Aβ toxicity in a *C. elegans* model for Aβ aggregation. As some seeding proteins may not be su□ciently abundant to be detected in disease aggregates, it will be relevant to investigate the other late-aggregating proteins as well as early-aggregating proteins that continue to become more insoluble with age. In particular, proteins associated with the eight pathways highlighted by the IPA analysis should be promising candidates. Furthermore, it is possible that post-translational modifications occurring with age influence the seeding ability of these proteins. A number of post-translational modifications have been identified in disease-associated aggregates, and a recent study detected carbonylation in the mouse age-dependent insoluble proteome (Tanase et al., 2016). Another possibility is that changes in the structure of insoluble proteins could occur with age and explain why they become e□ective seeds.

A pressing aspect to be addressed in future studies is to determine the seeding activity of age-dependent protein aggregation *in vivo*. This could be done by using established mouse models for Aβ seeding, in which past experiments showed that intracerebral injection of Aβ-rich brain extracts in young APP-transgenic mice induced Aβ plaque formation prior to the appearance of endogenous Aβ deposits (Meyer-Luehmann et al., 2006). Of note, it is unclear whether this *in vivo* model would be su□ciently sensitive to detect heterologous seeding by the age-dependent insoluble proteome within the time window preceding the emergence of endogenous plaques and cerebral amyloid-β angiopathy. Indeed, our *in vitro* data showed a reproducible but relatively low seeding activity compared to Aβ seeds and therefore successful *in vivo* seeding studies may require repeated injections of insoluble extracts and/or very long incubation times.

Together, the present findings emphasize the need to understand better why protein insolubility is prevalent in older age and how the cellular quality-control systems fail to prevent it. Abrogating the formation of heterologous seeds could significantly reduce disease-associated seeding events and delay the onset of AD.

AUTHOR CONTRIBUTIONS

NG, FB, DD: conceived experiments, NG, AB, KWL, FB, DD: performed experiments; NG, CH, PvN, FB: performed data analysis; NG, KWL, FB, DD: wrote manuscript; AS, MF: revised manuscript; MF: contributed essential reagents; AS, FB, DD: supervised project.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fnagi.2017.00138/full#supplementary-material

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