Published in final edited form as:

Exp Eye Res. 2010 July ; 91(1): 107–110. doi:10.1016/j.exer.2010.04.004.

Preliminary Quantitative Proteomic Characterization of Glaucomatous *in vivo* Rat Retinal Ganglion Cells

John W Crabb^{1,2,3}, Xianglin Yuan^{1,2}, Galina Dvorianchikova⁴, Dmitry Ivanov⁴, John S Crabb^{1,2}, and Valery I Shestopalov^{4,5}

¹Cole Eye Institute, Cleveland Clinic, Cleveland, OH

²Lerner Research Institute, Cleveland Clinic, Cleveland, OH

³Department of Ophthalmology, Cleveland Clinic Lerner College of Medicine of Case Western Reserve University, Cleveland, OH 44106

⁴Bascom Palmer Eye Institute, University of Miami Miller School of Medicine, Miami, FL

⁵Department of Cell Biology and Anatomy, University of Miami Miller School of Medicine, Miami, FL

Abstract

Quantitative proteomic analysis was pursued of retinal ganglion cells (RGCs) from rats with unilateral experimental glaucoma. RGCs were isolated from 22 animals by immunopanning after 8 weeks of sustained elevated intraocular pressure. Proteins were quantified by LC MS/MS iTRAQ technology. Of the 268 proteins quantified, ~8% appeared elevated and ~13% decreased in glaucomatous RGCs. Voltage-dependent anion channel protein 2, aldose reductase, and ubiquitin were among the significantly elevated proteins while prothymosin was among the significantly decreased. The results demonstrate the feasibility of identifying global proteomic differences in protein expression between *in vivo* purified glaucomatous and control RGCs.

Keywords

Experimental Glaucoma; Retinal Ganglion Cells; Quantitative Proteomics; iTRAQ Technology; Mass Spectrometry

Glaucoma is a leading cause of blindness worldwide (Quigley and Broman, 2006) with most forms of the disease related to advanced age, increased intraocular pressure (IOP) and subsequent damage to retinal ganglion cells (RGCs) and the optic nerve (Hollings and Graham, 1966). While genes and loci have been linked to various types of glaucoma (Wiggs, 2007; Wirtz and Samples, 2003), the function of glaucoma gene products are not well established and the molecular mechanisms of glaucoma remain poorly understood. Of particular significance to glaucoma progression are the molecular pathways responsible for the selective loss of RGCs (Quigley et al., 1995). RGCs are the only retinal neurons that project axons to

© 2010 Elsevier Ltd. All rights reserved.

Correspondence should be addressed to, John W Crabb PhD, Cole Eye Institute i31, Cleveland Clinic Foundation, 9500 Euclid Avenue, Cleveland OH 44195, Tel 216-445-0425, Fax 216-445-3670, crabbj@ccf.org, Valery I Shestopalov PhD, Bascom Palmer Eye Institute, Univ of Miami Miller Sch of Med, 1638 NW 10th Avenue, Miami, FL 33136, Tel 305-547-3680, Fax 305-547-3658, vshestopalov@med.miami.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

the visual cortex of the brain but only account for ~0.5% of the neurons in the retina (Blackshaw et al., 2004). The relatively small number of RGCs and the difficulty to purify these cells from the many other complex types of cells in the retina present major hurdles to *in vivo* molecular characterization studies. We have taken advantage of an experimental glaucoma model to generate rats exhibiting elevated IOP in one eye and normal IOP in the other (Levkovitch-Verbin et al., 2002) and used immunopanning to isolate highly purified RGCs (Meyer-Franke et al., 1995) and LC MS/MS iTRAQ technology to quantify proteins (Yuan et al., 2010). Although only low microgram amounts of protein were recovered, sufficient protein was obtained for quantitative proteomic analysis. While previous qualitative proteomic studies have provided insights to a variety of ocular disorders (Crabb et al., 2002; Ng et al., 2008; Zhan et al., 2008) including glaucoma (Bhattacharya et al., 2006; Bhattacharya et al., 2005; Tezel et al., 2005), this is the first global quantitative proteomic analysis of *in vivo* purified RGCs.

Experimental glaucoma ocular hypertension was induced in adult Sprague Dawley rats (8–9 month old) by unilateral laser photocoagulation of anterior angle tissues (Levkovitch-Verbin et al., 2002). IOP was monitored in both eyes using the TONOLAB retractable tonometer on the first day post-surgery and weekly thereafter. A second laser treatment was performed 2–3 weeks later if the IOP difference between the treated and untreated fellow eye was < 6mm Hg. Integral IOP accumulation was calculated as the difference in the average weekly IOP between experimental and control eyes. Non-responders and hyper-responders were removed from experimental groups 8 weeks post-surgery, when animals typically accumulated Integral IOP in the range of 300–400 mm Hg-days. Animals with ocular abnormalities such as hemorrhage and excessive inflammation were also excluded. Treatment of animals conformed to the Association for Research in Vision and Ophthalmology Resolution on Treatment of Animals in Research, and to institutional animal care guidelines.

Rat RGCs were isolated by a modified two-step immunopanning method (Ivanov et al., 2006; Meyer-Franke et al., 1995). Actinomycin D (5mg/ml, Sigma) was added to all solutions to block transcription during experimental procedures and total treatment time was shortened to 4 hours (Huusko et al., 2004). Briefly, whole retinas were incubated in papain (0.7 U/ml; Worthington) and L-cysteine (0.3 mg/ml; Sigma) in Neurobasal-A medium (Invitrogen) for 30 min (37°C; CO₂ incubator), rinsed twice in 5 ml of medium, and triturated in 4 ml of the medium containing 1:50 B27 supplement (Invitrogen). Macrophage and endothelial cells were removed from the cell suspension by panning with anti-macrophage antiserum (Axell Accurate Chemical Corp.). RGCs were specifically bound to the panning plates with anti-Thy1.1 antibody, and unbound retinal cells were removed by washing with BSS media. Purified RGCs were released by trypsin incubation and frozen at –80°C until analysis. To help assess the purity of isolated RGCs, a few additional animals were injected with 2.1 µl of 5% 4Di-10Asp (DiA) (Invitrogen) in dimethylformamide in the superior cullicular region of the brain to produce highly specific RGC fluorescent labeling (Pavlidis et al., 2000). Rats were sacrificed 10 days after dye injection and RGCs were isolated.

RGCs were purified 8–9 weeks following the first laser treatment as soon as rats (n = 22) accumulated a mean Integral IOP exposure of 369 ± 70 mm Hg days. Average IOP is shown in Figure 1C for experimental and control eyes over time. Animals with high (>50 mmHg) IOP elevation and non-responders were excluded from the analysis. Individual retinas yielded on average $28 \pm 15 \times 10^3$ RGCs per normotensive control eye and $22 \pm 10 \times 10^3$ per laser treated eye. Neuronal loss in the ganglion cell layer was assessed by confocal microscopy and immunostaining for NeuN in randomly sampled 1/8 sectors of experimental (n=10) and control retinas (n=10) and was found to average ~40% in treated eyes (Fig 1AB). Additional sectors (1/8–1/10) of the same retinas were sampled and used for total RNA extraction to probe by PCR for RGC contamination with other cell types. The purity of the isolated RGCs was evaluated in a separate experiment by retrograde fluorescent labeling of RGCs with DiA, which

showed about 95% of the cells double-labeled for DiA and DAPI (Fig 1C), in good agreement with others (Wang et al., 2007). The RGC preparations were routinely tested for purity, first, by double-staining with anti-NeuN antibodies specific for neurons and with DAPI to detect all cells. Second, quantitative RT-PCR (Fig 1D) was used to measure relative abundance (normalized to β -actin) of transcripts specific to other retinal cell types in the total RNA extracted from the RGC preparations (Ivanov et al., 2006). In contrast to the RGC-associated *Thy1* transcript and the general neuronal marker *Mapt*, other cell type markers (*Rho*, *Cd11b*, *Cd68*, and *Gfap*) were not detected (Fig 1E). These data support the high purity (> 90%) of the RGCs used for proteomic analysis.

Purified RGCs were homogenized in triethylammonium bicarbonate buffer containing SDS and soluble protein was extracted. From 22 animals, two pooled protein samples were prepared, one containing protein from the glaucomatous RGCs and the other from control RGCs isolated from untreated eyes. The pooled samples were quantified by phenylthiocarbamyl amino acid analysis (Crabb et al., 1997) and ~5 μ g protein of each used for proteomic analysis (the maximum available from laser-treated eyes). Protein was reduced, alkylated and digested with trypsin (Zhan et al., 2008) and peptides were labeled with amine specific iTRAQ (isobaric tag for relative and absolute quantitation) tags as described previously (Yuan et al., 2010). Peptides from the glaucomatous sample were labeled with iTRAQ tag 117 and mixed with an equal amount of control peptides labeled with iTRAQ tag 115. The iTRAQ-labeled peptide mixture was fractionated by strong cation exchange (SCX) chromatography and 1 min fractions collected and vacuum dried (Ng et al., 2008). SCX fractions (n = 60) were chromatographed on a PepMap100 C18 column (75 μ m $\times$ 15 cm) using a Ultimate 3000 LC system (LC Packings) and aqueous trifluoroacetic acid/acetonitrile solvents and using a Probot target spotter (LC Packings), the eluant was directly mixed with matrix [1:2 (v/v), 4.5 mg/ml α -cyano-4-hydroxycinnamic acid in 0.56 mM ammonium citrate] and spotted on a MALDI target. Peptide analysis by MALDI TOF TOF tandem mass spectrometry was performed with a model 4800 instrument (Applied Biosystems) using 2000 laser shots averaged per MS/MS spectrum (1kV collision energy), a maximum of 20 MS/MS spectra collected per target spot and a minimum signal-to-noise of 35 (Yuan et al., 2010). Instrument operation and data acquisition/analysis utilized GPS Explorer 3.6 software (Applied Biosystems); protein identification from MS/MS data utilized Mascot 2.1 and the Swiss Protein database version 56.2. Database searches were restricted to tryptic peptides of rat sequences (7180 sequence entries) and mouse sequences (15980 sequence entries) with 2 missed tryptic cleavage sites allowed, precursor ion mass tolerance = 0.8 Da, and fragment ion mass tolerance = 0.8 Da. Fixed modifications were selected for iTRAQ N-terminal and ϵ -Lys modifications and S-methyl Cys; Met oxidation was used as a variable modification.

Protein quantification from iTRAQ-labeling utilized GPS Explorer 3.6 and custom code written in the statistical program R (<http://www.lerner.ccf.org/eye/crabb/software/>) (Yuan et al., 2010). Minimum criteria for quantitation included at least two unique peptides per identified protein, a 95% confidence interval for the Mascot protein score and Mascot peptide ion scores ≥ 25 . Peptide ratios were converted to natural logs and outlier peptide ratios were eliminated using Dixon's test (Rorabacher, 1991) and the interquartile range function in R with k = 1.75. Peptides assigned to more than one protein by the Mascot search program were restricted to the highest scoring protein during analysis in R. After deleting outliers and multiple copies of the same peptide, peptide ratios were normalized to the sample peptide median [i.e., normalized peptide ratio = unnormalized peptide ratio/median] prior to calculating mean protein ratios, standard deviation (SD) and p values (2-sided t-test). Protein ratios were determined in log space and transformed for reporting.

A total of 268 proteins were quantified with two or more peptides (Supplementary Table 1) with an additional 239 proteins detected by a single peptide (not reported). In support of the

quality of the cell preparations, γ -synuclein, a marker of RGCs (Surgucheva et al., 2008), was among the quantified proteins. No known glaucoma associated gene products were detected. Of the 268 proteins, a majority (~78%) exhibited protein ratios near the mean ratio of 1.0, indicating approximately equal amounts in the glaucomatous and control RGCs. Using The Protein Analysis THrough Evolutionary Relationships (PANTHER) Classification System (www.pantherdb.org), a variety of biological processes can be associated with the quantified proteins, with a majority involved with cellular metabolism (~50%), signal transduction (~6.9%), intracellular protein traffic (5.2%) and cell cycle (5%). Bioinformatic analysis of the 268 proteins using Ingenuity Pathways Analysis 8.0 (Ingenuity® Systems) assigned RNA post-translational modification, small molecule biochemistry and energy production as the highest scoring pathway networks.

About ~8% of the quantified proteins (n = 22) exhibited ratios ≥ 1 SD above the mean and ~13% (n = 36) exhibited ratios ≥ 1 SD below the mean. Three proteins were significantly more abundant in glaucomatous RGCs, namely (i) voltage-dependent anion channel protein 2, a regulator of ion transport in the mitochondrial outer membrane; (ii), aldose reductase, a cytoplasmic NADPH-dependent oxidoreductase associated with signaling and cell proliferation pathways; and (iii), and ubiquitin, a cytoplasmic and nuclear protein modifier that generates many functional consequences, including proteasomal degradation and regulation of gene expression. One protein was significantly less abundant in glaucomatous RGCs, namely prothymosin α , a nuclear protein that is covalently modified with RNA and may mediate immune functions in anti-apoptosis and cell proliferation pathways. These proteins plus others with ratios ≥ 2 SD above or below the mean are listed in Table 1 as candidate proteins for differential expression in glaucomatous RGCs. Further studies are required to validate protein expression changes associated with glaucoma.

This preliminary study demonstrate the feasibility of unbiased, global quantitative proteomic analysis of purified RGCs using LC MS/MS iTRAQ technology. This approach is distinctly different than that employed in the only other proteomic study to date of enriched RGCs where gel staining and immunoblot were used to study a single protein family (Yang et al., 2008). Furthermore, direct proteomic profiling offers advantages over gene expression analyses as only ~20% correlation has been found between transcript and cognate protein levels (Tian et al., 2004). Global quantitative proteomic analysis of purified RGCs offers high potential for future insights into the mechanisms of glaucoma pathology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Supported by NIH grants EY017991, P30 EY014801, EY018147, EY14239, EY15638, Ohio BRTT grant 05-29, unrestricted grants from Research to Prevent Blindness (RPB) to the Cole Eye Institute, Cleveland Clinic and to the Department of Ophthalmology, U. Miami, an RPB Senior Investigator Award, and a Steinbach Award.

References

- Bhattacharya SK, Crabb JS, Bonilha VL, Gu X, Takahara H, Crabb JW. Proteomics implicates peptidyl arginine deiminase 2 and optic nerve citrullination in glaucoma pathogenesis. *Invest Ophthalmol Vis Sci* 2006;47:2508–2514. [PubMed: 16723463]
- Bhattacharya SK, Rockwood EJ, Smith SD, Bonilha VL, Crabb JS, Kuchtey RW, Robertson NG, Peachey NS, Morton CC, Crabb JW. Proteomics reveal Cochlin deposits associated with glaucomatous trabecular meshwork. *J Biol Chem* 2005;280:6080–6084. [PubMed: 15579465]

- Blackshaw S, Harpavat S, Trimarchi J, Cai L, Huang H, Kuo WP, Weber G, Lee K, Fraioli RE, Cho SH, Yung R, Asch E, Ohno-Machado L, Wong WH, Cepko CL. Genomic analysis of mouse retinal development. *PLoS Biol* 2004;2:E247. [PubMed: 15226823]
- Crabb JW, Miyagi M, Gu X, Shadrach K, West KA, Sakaguchi H, Kamei M, Hasan A, Yan L, Rayborn ME, Salomon RG, Hollyfield JG. Drusen proteome analysis: an approach to the etiology of age-related macular degeneration. *Proc Natl Acad Sci U S A* 2002;99:14682–14687. [PubMed: 12391305]
- Crabb JW.; West, KA.; Dodson, WS.; Hulmes, JD. Amino Acid Analysis. In: Coligan, JE.; Ploegh, HL.; Smith, JA.; Speicher, DW., editors. *Current protocols in protein science*. Vol. Unit 11.9. John Wiley & Sons, Inc; 1997. p. 11.09.01-11.09.42.
- Hollows FC, Graham PA. Intra-ocular pressure, glaucoma and Glaucoma suspects in defined population. *Br J Ophthalmol* 1966;50:570–586. [PubMed: 5954089]
- Huusko P, Ponciano-Jackson D, Wolf M, Kiefer JA, Azorsa DO, Tuzmen S, Weaver D, Robbins C, Moses T, Allinen M, Hautaniemi S, Chen Y, Elkahouloun A, Basik M, Bova GS, Bubendorf L, Lugli A, Sauter G, Schleutker J, Ozcelik H, Elowe S, Pawson T, Trent JM, Carpten JD, Kallioniemi OP, Mousses S. Nonsense-mediated decay microarray analysis identifies mutations of EPHB2 in human prostate cancer. *Nat Genet* 2004;36:979–983. [PubMed: 15300251]
- Ivanov D, Dvorianchikova G, Nathanson L, McKinnon SJ, Shestopalov VI. Microarray analysis of gene expression in adult retinal ganglion cells. *FEBS Lett* 2006;580:331–335. [PubMed: 16376886]
- Levkovitch-Verbin H, Quigley HA, Martin KR, Valenta D, Baumrind LA, Pease ME. Translimbal laser photocoagulation to the trabecular meshwork as a model of glaucoma in rats. *Invest Ophthalmol Vis Sci* 2002;43:402–410. [PubMed: 11818384]
- Meyer-Franke A, Kaplan MR, Pfrieger FW, Barres BA. Characterization of the signaling interactions that promote the survival and growth of developing retinal ganglion cells in culture. *Neuron* 1995;15:805–819. [PubMed: 7576630]
- Ng KP, Gugiu B, Renganathan K, Davies MW, Gu X, Crabb JS, Kim SR, Rozanowska MB, Bonilha VL, Rayborn ME, Salomon RG, Sparrow JR, Boulton ME, Hollyfield JG, Crabb JW. Retinal pigment epithelium lipofuscin proteomics. *Mol Cell Proteomics* 2008;7:1397–1405. [PubMed: 18436525]
- Pavlidis M, Fischer D, Thanos S. Photoreceptor degeneration in the RCS rat attenuates dendritic transport and axonal regeneration of ganglion cells. *Invest Ophthalmol Vis Sci* 2000;41:2318–2328. [PubMed: 10892879]
- Quigley HA, Broman AT. The number of people with glaucoma worldwide in 2010 and 2020. *Br J Ophthalmol* 2006;90:262–267. [PubMed: 16488940]
- Quigley HA, Nickells RW, Kerrigan LA, Pease ME, Thibault DJ, Zack DJ. Retinal ganglion cell death in experimental glaucoma and after axotomy occurs by apoptosis. *Invest Ophthalmol Vis Sci* 1995;36:774–786. [PubMed: 7706025]
- Rorabacher DB. Statistical treatment for rejection of deviant values: critical values of Dixon's "Q" parameter and related subrange ratios at the 95% confidence interval. *Anal Chem* 1991;63:139–146.
- Surgucheva I, Shestopalov VI, Surguchov A. Effect of gamma-synuclein silencing on apoptotic pathways in retinal ganglion cells. *J Biol Chem* 2008;283:36377–36385. [PubMed: 18936092]
- Tezel G, Yang X, Cai J. Proteomic identification of oxidatively modified retinal proteins in a chronic pressure-induced rat model of glaucoma. *Invest Ophthalmol Vis Sci* 2005;46:3177–3187. [PubMed: 16123417]
- Tian Q, Stepaniants SB, Mao M, Weng L, Feetham MC, Doyle MJ, Yi EC, Dai H, Thorsson V, Eng J, Goodlett D, Berger JP, Gunter B, Linseley PS, Stoughton RB, Aebersold R, Collins SJ, Hanlon WA, Hood LE. Integrated genomic and proteomic analyses of gene expression in Mammalian cells. *Mol Cell Proteomics* 2004;3:960–969. [PubMed: 15238602]
- Wang JT, Kunzevitzky NJ, Dugas JC, Cameron M, Barres BA, Goldberg JL. Disease gene candidates revealed by expression profiling of retinal ganglion cell development. *J Neurosci* 2007;27:8593–8603. [PubMed: 17687037]
- Wiggs JL. Genetic etiologies of glaucoma. *Arch Ophthalmol* 2007;125:30–37. [PubMed: 17210849]
- Wirtz, MK.; Samples, JR. Glaucoma Genetics. In: Morrison, JC.; Pollack, IP., editors. *Glaucoma: Science and Practice*. New York: Thieme; 2003. p. 12-21.

- Yang X, Luo C, Cai J, Pierce WM, Tezel G. Phosphorylation-dependent interaction with 14-3-3 in the regulation of bad trafficking in retinal ganglion cells. *Invest Ophthalmol Vis Sci* 2008;49:2483–2494. [PubMed: 18296656]
- Yuan X, Gu X, Crabb JS, Yue X, Shadrach K, Hollyfield JG, Crabb JW. Quantitative Proteomic Comparison of the Macular Bruch's Membrane/Choroid Complex from Age-related Macular Degeneration and Normal Eyes. *Mol Cell Proteomics*. 2010 (Epub ahead of print) PMID 20177130.
- Zhan X, Du Y, Crabb JS, Gu X, Kern TS, Crabb JW. Targets of Tyrosine Nitration in Diabetic Rat Retina. *Mol Cell Proteomics* 2008;7:864–874. [PubMed: 18165258]

Figure 1. Rat Retinas and Isolated RGCs

Elevated IOP was induced unilaterally in rats by laser photocoagulation of anterior angle tissue, resulting in experimental glaucoma and retinal damage. **A.** Representative confocal micrographs showing neuronal density in the ganglion cell layer (GCL) of control and glaucomatous rat retinas taken at the same eccentricity from the optic nerve. Neurons in the GCL are labeled with anti-NeuN antibodies (green). Bar, 50 μ m. **B.** Neuronal cell quantification (NeuN⁺ cells/standard field) in the ganglion cell layer performed by confocal microscopy revealed approximated 40% cell loss in laser treated (red) relative to fellow control retinas. **C.** The average IOP in experimental (red) and control (blue) eyes from the 22 animals used for RGC purification and proteomics analysis; astericks indicate laser treatments. **D.** Micrographs of RGCs isolated from naïve eyes that were retrogradely labeled *in vivo* with DiA (green) ten days prior to harvesting the retina; nuclei are counter-stained with DAPI (cyan). Single cell lacking DiA is indicated by arrow. Bar, 25 μ m. **E.** To assess contamination with other types of retinal cells, the relative abundance of marker gene transcripts for RGC-associated *Thy1*, general neuronal marker *Mapt*, *Rho* (for photoreceptors), *Gfap* (for astrocytes and Müller cells), *Cd11b* (for microglia) and *Cd68* (for leucocytes) was measured by quantitative RT-PCR and normalized to β -actin (*Actb*). All analyzed cell markers were detected at significantly lower levels than *Thy1*.

Table 1
Candidate Proteins for Differential Expression in Glaucomatous Rat Retinal Ganglion Cells

SwissProt Accession	Protein	Protein Ratio	SD	P value	Unique Peptides	Sequence Coverage
<i>Apparently Elevated Proteins</i>						
Q3ER34	Aconitate hydratase, mitochondrial	1.95*	NA	NA	2	3.3%
P52555	Endoplasmic reticulum protein ERp29	1.79*	NA	NA	2	9.6%
Q9WV25	Poly(U)-binding-splicing factor PUF60	1.33*	NA	NA	2	2.7%
P81155	Voltage-dependent anion-selective channel protein 2	1.23	0.17	0.034	6	24.1%
P07943	Aldose reductase	1.20	0.02	0.006	3	8.5%
P62989	Ubiquitin	1.20	0.12	0.009	7	77.6%
<i>Apparently Decreased Proteins</i>						
Q8R4E6	Purine-rich element-binding protein gamma	0.70*	NA	NA	2	3.7%
O88453	Scaffold attachment factor B1	0.69*	NA	NA	2	1.5%
P06302	Prothymosin alpha	0.66*	0.16	0.015	4	13.4%
P54313	Guanine nucleotide-binding protein (Transducin beta)	0.64*	NA	NA	2	5.0%
Q04462	Valyl-tRNA synthetase	0.63*	NA	NA	2	1.3%
O35331	Pyridoxal kinase	0.41*	NA	NA	2	9.3%

Proteins at least 2 SD from the mean ratio (denoted by astericks) and proteins with p values < 0.05 at least 1 SD from the mean ratio are shown (from Supplemental Table 1, mean ratio = 1.0). Protein ratio = Glaucomatous/control; SD = standard deviation; p values are from the two sided t-test for the null hypothesis that the peptide mean = 1.0. NA, not applicable because < 3 peptides.